BIOCHEMICAL PHARMACOLOGY 77 (2009) 826–834

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Activation and modulation of 72 kDa matrix metalloproteinase-2 by peroxynitrite and glutathione

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ARTICLE INFO

Article history:
Received 30 September 2008
Accepted 4 November 2008

Keywords:
Matrix metalloproteinase-2
Oxidative stress
Peroxynitrite
S-glutathiolation
Troponin I

ABSTRACT

Matrix metalloproteinase-2 (MMP-2) has emerged as a key protease in various pathologies associated with oxidative stress, including myocardial ischemia-reperfusion, heart failure or inflammation. Peroxynitrite ($ONOO^-$), an important effector of oxidative stress, was reported to activate some full length MMP zymogens, particularly in the presence of glutathione (GSH), but whether this occurs for MMP-2 is unknown. Treating MMP-2 zymogen with $ONOO^-$ resulted in a concentration-dependent regulation of MMP-2, with 0.3–1 μM $ONOO^-$ increasing and 30–100 μM $ONOO^-$ attenuating enzyme activity. The enzyme's V_{max} was also significantly increased by 1 μM $ONOO^-$. Comparable responses to $ONOO^-$ treatment were observed using the intracellular target of MMP-2, troponin I (TnI). GSH at 100 μM attenuated the effects of $ONOO^-$ on MMP-2. Mass spectrometry revealed that $ONOO^-$ can oxidize and, in the presence of GSH, S-glutathiolate the MMP-2 zymogen or a synthetic peptide containing the cysteine-switch motif in the enzyme's autoinhibitory domain. These results suggest that $ONOO^-$ and GSH can modulate the activity of 72 kDa MMP-2 by modifying the cysteine residue in the autoinhibitory domain of the zymogen, a process that may be relevant to pathophysiological conditions associated with increased oxidative stress.

1. Introduction

There is increasing evidence that matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are implicated in a number of pathological conditions including neurodegenerative diseases [1] and ischemia-reperfusion injury in the heart [2–4]. MMP-2 has recently emerged as a key enzyme involved in several cardiac conditions associated with enhanced oxidative stress [2–6]. We found that MMP-2 activation results in the degradation of specific proteins, including troponin I (TnI) [7], myosin light chain 1 [8] and α-actinin [9], and thus may be responsible, at least in part, for cardiac dysfunction in ischemia-reperfusion injury [2].

MMPs are expressed in cells as catalytically inactive zymogens (''proMMPs'') that are maintained in a latent form through an interaction between a conserved cysteine (Cys) residue in the autoinhibitory domain and a zinc ion in the catalytic site. The best characterized activation of MMPs occurs

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Abbreviations: APMA, 4-aminophenylmercuric acetate; DPN, decomposed peroxynitrite; DTT, dithiothreitol; ESI-QTOF, electrospray ionization-quadrupole-time of flight tandem mass spectrometry; GSH, glutathione; IAM, iodoacetamide; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MMP, matrix metalloproteinase; $ONOO^-$, peroxynitrite; TnI, troponin I.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.11.004

during their secretion into the extracellular space, with proteolytic removal of the autoinhibitory domain and subsequent exposure of the catalytic site [5,6,10]. A non-proteolytic activation of MMPs has been postulated to occur by the covalent modification of the Cys residue in the autoinhibitory domain by effectors of oxidative stress, including the endogenous oxidant peroxynitrite ($ONOO^-$), but the mechanism by which $ONOO^-$ can activate MMPs is not clearly understood. $ONOO^-$ has been reported to maximally activate MMP-1, -8 and -9 in the presence of equimolar concentrations of glutathione (GSH) by a reaction that would produce a glutathione disulfide S-oxide adduct at the conserved Cys residue in the autoinhibitory domain [11]. Considering that MMP-2 is ubiquitously expressed and that it is the most abundant MMP in the heart, the elucidation of the activation mechanism by $ONOO^-$ is of particular interest. Consequently, the present study investigated how $ONOO^-$ interacts with 72 kDa MMP-2 zymogen in the absence or presence of GSH.

2. Materials and methods

2.1. Materials

All reagents were purchased from EM Science (Gibbstown, NJ) or Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Human recombinant 72 kDa MMP-2 was purchased from Calbiochem (La Jolla, CA). Human recombinant TnI in 50 mM Tris pH 7.6 containing 0.5 M NaCl, 0.1 mM DTT and 0.1% (w/v) NaN_3, was a gift from Dr. James Potter (Department of Molecular and Cellular Pharmacology, University of Miami, FL). The KPRCGNPDVANYNFFPR synthetic peptide, corresponding to the amino acids 99–115 and containing the cysteine-switch motif of human MMP-2 zymogen, was obtained from the Peptide Service of the University of Calgary (Calgary, Alberta, Canada).

2.2. Preparation of peroxynitrite

$ONOO^-$ and decomposed $ONOO^-$ (DPN) were prepared as previously described [12]. Stock solutions of $ONOO^-$ were stored at −80 °C, and prior to each experiment the concentration was determined spectrophotometrically ($\varepsilon^{302} = 1.67\ mM^{-1}\ cm^{-1}$).

2.3. 72 kDa MMP-2 treatment

Human recombinant 72 kDa MMP-2 (28 nM) was diluted in the absence or presence 100 μM GSH in 50 mM Tris buffer pH 7.6 at 37 °C. The concentration of GSH was chosen from concentrations (10 μM–10 mM) tested in preliminary experiments and agrees with those found to modulate the effect of $ONOO^-$ on other proteins [11,13]. Due to its short half-life at physiological pH, $ONOO^-$ (0.3–100 μM) was added in some experiments as a series of three additions at 5 min intervals.

2.4. DQ-gelatin degradation

$ONOO^-$-treated 72 kDa MMP-2 was incubated in triplicate (120 μl final volume) in a black polystyrene, 96-well half-area plate (Corning Inc., Corning, NY) with 10 μg/well DQ-gelatin (Molecular Probes, Eugene, OR) in 10 mM HEPES pH 7.5, 150 mM NaCl, 5.0 mM $CaCl_2$, 0.1% Triton X-100 and 5 μg/ml NaN_3. The increase in fluorescence (λ_{ex} 495 nm, λ_{em} 515 nm) due to DQ-gelatin proteolytic degradation was monitored in a Fluoroskan Ascent plate reader (Thermolabsystem, Waltham, MA) at 37 °C every 30 min.

2.5. OmniMMP® degradation

The hydrolysis of 2.5–30 μM OmniMMP® fluorogenic peptide (Biomol International, Plymouth Meeting, PA), prepared in 1.8% (v/v) DMSO, by $ONOO^-$-treated 72 kDa MMP-2 (2.5 nM final concentration) in 50 mM Tris pH 7.6, 10 mM $CaCl_2$, 0.05% Brij-35, 10 μM $ZnSO_4$ was measured every 30 s for 1 h (λ_{ex} 328 nm, λ_{em} 393 nm) at 37 °C using a SPECTRAmax Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA). 100 μM 4-aminophenyl-mercuric acetate (APMA, maximal activation) and DPN (background activation) were used as comparison points for the activation of MMP-2 by $ONOO^-$. Assays were made in 120 μl total volume in black polystyrene half-area plates, and contained MMP-2 and 15 μM substrate or DMSO vehicle (1.8%, v/v). The reaction rates were determined by linear regression of the experimental data ($r^2 > 0.98$) using SOFTmax Pro 4.8 (Molecular Devices, Sunnyvale, CA). Appropriate lag times, to preclude data obtained prior to equilibration at 37 °C, and end times, to preclude data obtained following loss of linearity, were entered manually prior to linear regression. The reaction rates were corrected for fluorescence background. K_M and V_{max} values were obtained from Michaelis–Menten plots using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA).

2.6. Gelatin zymography

72 kDa MMP-2 was diluted with non-reducing Laemmli buffer (0.5 M Tris pH 6.8, 30% glycerol, 10% (w/v) SDS, 0.012% (w/v) bromophenol blue) and electrophoresed on an 8% polyacrylamide gel copolymerized with 2 mg/ml gelatin. The gel was rinsed in 2.5% (v/v) Triton X-100, incubated for 16 h in 50 mM Tris buffer containing 5 mM $CaCl_2$, 150 mM NaCl, and 0.05% NaN_3, stained with 0.05% Coomassie blue in 10% acetic acid containing 25% (v/v) methanol, and destained in 8% (v/v) acetic acid containing 4% (v/v) methanol.

2.7. Troponin I degradation

$ONOO^-$-treated 72 kDa MMP-2 was incubated for 40 min at 37 °C with 2.0 μM TnI in 50 mM Tris buffer containing 5 mM $CaCl_2$, 150 mM NaCl and 0.05% (w/v) NaN_3 (DTT < 1 μM). Samples were diluted in reducing Laemmli buffer (containing 6% (v/v) β-mercaptoethanol) [14], boiled for 5 min, run on a 15% SDS-PAGE gel, and stained with Coomassie blue. The appearance of lower molecular weight fragments below the band for TnI (25 kDa) indicated TnI degradation.

2.8. Mass-spectrometry analysis

The synthetic peptide KPRCGNPDVANYNFFPR (10 μM), identical to the tryptic fragment of human 72 kDa MMP-2 containing the cysteine-switch motif, was incubated with

1 μM, 10 μM or 100 μM $ONOO^-$ at 37 °C for 15 min in 50 mM Tris pH 8.2 or pH 7.6 in the absence or presence of 100 μM GSH. To block the sulfhydryl group of Cys prior to the addition of $ONOO^-$, some samples were treated with 20 μM iodoacetamide (IAM) for 30 min at room temperature. The 72 kDa MMP-2 (0.1 μM) was incubated with 10 or 100 μM $ONOO^-$ for 30 min at 37 °C in 50 mM Tris pH 7.6 in the presence or absence of 100 μM GSH. Mass spectra were obtained after in-solution trypsin digestion, under native conditions [15]. Mass spectrometry was performed on an UltraFlex TOF/TOF (Bruker Daltonics, Billerica, MA) or a LC-ESI-QqTOF mass spectrometer (Waters, Milford, MA). The MALDI-TOF MS used a 50 Hz pulsed 337 nm nitrogen laser, operating in reflectron (positive ion) mode. Samples were applied as a mixture of 1.0 μl reaction solution and 1.0 μl of 4 mg/ml α-cyano-4-hydroxycinnamic acid in 2:1 (v/v) acetonitrile–water mixture containing 2.5% trifluoroacetic acid (MALDI-TOF), or diluted in 0.1% formic acid prior to injection (ESI-QqTOF). A standard peptide mixture (Bruker Daltonics, Billerica, MA) for MALDI-TOF, or [Glu^1]-fibrinopeptide B and leucine enkephalin (Sigma, St. Louis, MO) for ESI-QqTOF were used for calibration. MS/MS fragments were generated in post-source decay mode, selecting the precursor mass with a 2 Da window. Monoisotopic masses were obtained by post-processing of the MS spectra using FlexAnalysis 2.4 (Bruker Daltonics, Billerica, MA) and differed by ±0.4 Da from calculated masses. The Waters QTOF Premier MS was equipped with an Acquity UPLC system (Waters, Milford, MA) and an Atlantis dC18 column (4.6 mm × 150 mm, 5 μm). The quadrupole operated in a survey scan for data direct analysis mode, and TOF analyzer operated in positive, reflectron mode. Following column equilibration with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), a gradient elution was run from 1% to 15% solvent B for 5.0 min followed by 15–35% solvent B for 30 min, at a flow rate of 0.35 μl/min. All mass spectrometry experiments were performed in triplicate.

2.9. Bioinformatics analysis

Protein Prospector (http://prospector.ucsf.edu/prospector/4.27.1) was used for in silico trypsin digestion of human MMP-2 (accession no. P08253) using the parameters: 1 missed cleavage and ±0.2 Da mass tolerance, carbamidomethylation and reduction of Cys residues, variable oxidation of methionines. The Mascot (http://www.matrixscience.com) MS/MS ion search was used to search mass spectra against SwissProt database using the parameters: up to two missed cleavages; ±0.2 Da mass tolerance; Cys nitrosylation, di- and tri-oxidation; Tyr and Phe oxidation and dioxidation; Tyr nitration. The QTOF MS/MS spectra were searched against SwissProt database using Peaks Studio version 4.5 (Bioinformatics Systems, Waterloo, Ontario, Canada).

2.10. Statistical analysis

Data are expressed as means ± SE. Comparison among multiple groups was performed using one- or two-way analysis of variance (ANOVA) followed by post-hoc tests. Two-tailed p values <0.05 were considered statistically significant.

3. Results

3.1. Degradation of fluorogenic substrates by $ONOO^-$-treated 72 kDa MMP-2

The purity of 72 kDa MMP-2 was estimated by mass spectrometry and gelatin zymography showing minimal contribution of the 64 kDa MMP-2 form (Fig. S1). Using the DQ-gelatin degradation assay, the greatest stimulatory effect of all concentrations of $ONOO^-$ tested (0.3–100 μM) was seen with 0.3 μM $ONOO^-$ (Fig. 1A, left panel) compared to DPN-treated or untreated enzyme (not shown as it overlaps with the DPN curve). MMP-2 activity was inhibited by 100 μM $ONOO^-$ (Fig. 1A). The addition of 100 μM GSH prior to $ONOO^-$ treatment resulted in highest enzyme activation with 10 μM $ONOO^-$ and a reduced attenuation of activity with 100 μM $ONOO^-$ (Fig. 1A, right panel). The initial rate of DQ-gelatin degradation was significantly higher at 0.3 μM (98 ± 3.2 RFU/min) and 10 μM (78 ± 1.8 RFU/min), but lower at 100 μM (20 ± 0.4 RFU/min) $ONOO^-$ compared to that obtained with DPN (67 ± 2.3 RFU/min). In the presence of 100 μM GSH the rate was significantly higher with 10 μM (71 ± 3.2 RFU/min) and lower after 100 μM (41 ± 0.4 RFU/min) $ONOO^-$ compared to DPN control (60 ± 2.0 RFU/min). Addition of GSH alone did not activate 72 kDa MMP-2 (data not shown).

The OmniMMP assay was used to determine the V_{max} (indicative of enzyme's catalytic turnover) and K_M (indicative of enzyme's substrate affinity) values for the degradation of a fluorogenic substrate by $ONOO^-$-treated MMP-2. Compared to 100 μM APMA (an organomercuric compound that specifically alkylates Cys residues and maximally activates the enzyme), the highest enzyme activation with $ONOO^-$ was observed at 1 μM (approx. 10% of the maximal activation achieved by APMA) (Fig. S2). In the absence of GSH, a bell-shaped dependence of V_{max} on $ONOO^-$ concentration and a two-fold increase of V_{max} at 1 μM $ONOO^-$, when compared to DPN, were observed (Fig. 1B). Treating 72 kDa MMP-2 with higher $ONOO^-$ concentrations resulted in the inhibition of enzyme activity. GSH attenuated $ONOO^-$-induced activation of 72 kDa MMP-2. The K_M values for OmniMMP degradation by MMP-2 were not significantly changed by $ONOO^-$ in the absence or presence of 100 μM GSH (data not shown).

3.2. Troponin I degradation by $ONOO^-$-treated 72 kDa MMP-2

We previously showed that 64 kDa MMP-2 cleaves TnI [7,16]. $ONOO^-$-treated 72 kDa MMP-2 degraded TnI, as evidenced by the appearance of degradation products around 22 kDa and 18 kDa and a decrease in the band intensity of the intact TnI, which peaked at 3 μM $ONOO^-$ (Fig. 2). In the presence of 100 μM GSH, the apparent maximal degradation of TnI by $ONOO^-$-treated MMP-2 occurred at 10 μM (Fig. 2).

3.3. Mass spectrometry analysis

MALDI-MS/MS analysis of the synthetic KPRCGNPDVANYNFFPR peptide, predicted by in silico trypsin digestion (Table S1), identified the y and b ion series and the correct amino acid sequence, as verified by SwissProt and NCBInr database search (Fig. S3).

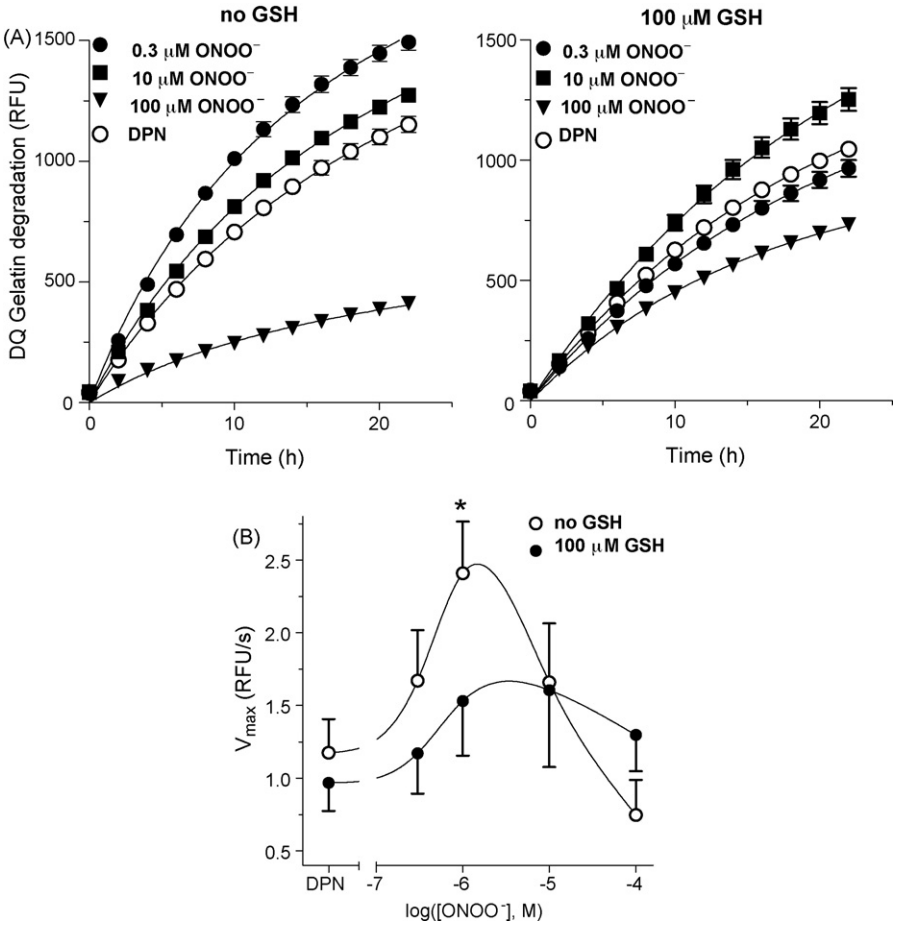

Fig. 1 – Degradation of fluorogenic substrates by $ONOO^-$-treated 72 kDa MMP-2. (A) DQ-gelatin degradation by $ONOO^-$-treated 72 kDa MMP-2 in the absence or presence of 100 μM GSH. (B) Effect of $ONOO^-$ on 72 kDa MMP-2's V_{max} in the absence or presence of 100 μM GSH. 72 kDa MMP-2 was treated with 0.3–100 μM $ONOO^-$ or decomposed $ONOO^-$ (DPN) and incubated with 2.5–30 μM OmniMMP. Data represent means ± SEM of three replicates. *Significant difference from DPN, $P < 0.05$ (repeated one-way ANOVA with Dunnett's post-hoc test).

The reaction of the peptide with $ONOO^-$ resulted in a concentration-dependent formation of higher molecular weight products (Fig. 3A). The most intense peaks appearing at 2026.92 Da and 2042.93 Da corresponded to a mass difference (Δm) of 47.99 Da and 31.98 Da, respectively, over the parent peptide (1994.96 Da), suggesting the incorporation of two (theoretical mass 31.99 Da) or three (theoretical mass 47.98 Da) oxygen atoms. At 100 μM $ONOO^-$, additional peaks at 2058.93 Da, 2074.92 Da and 2145.92 Da corresponding to Δm of 63.98 Da, 79.97 Da and 151.05 Da, respectively, were observed. The first two peaks had Δm values that correspond to the incorporation of four or five oxygen atoms, respectively. The latter peak is likely a Cys adduct since prior alkylation of this residue with iodoacetamide ablated the peak formation (see below). The symmetrical disulfide peptide of the parent peptide (calculated mass 3987.90 Da) was not observed following $ONOO^-$ treatment, indicating that the Cys in the parent peptide is inaccessible for dimerization. Liquid chromatography coupled with ESI-QqTOF MS/MS analysis of the peptide treated with $ONOO^-$ confirmed the oxidation of the Cys residue to SO_2H and SO_3H (Fig. S4). The oxidation of the tyrosine residue was also detected at higher $ONOO^-$-concentrations and pH, similar to that used by Okamoto et al. [17] (Table 1).

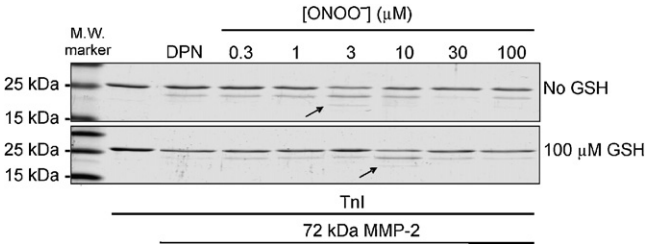

Fig. 2 – Troponin I (TnI) degradation by $ONOO^-$-treated 72 kDa MMP-2 in the absence or presence of 100 μM GSH. Representative Coomassie blue stained SDS-PAGE gel for $ONOO^-$-treated 72 kDa MMP-2 incubated with 2 μM TnI ($n = 3$ experiments with similar results). Arrows indicate $ONOO^-$ concentrations at which extensive TnI degradation was observed.

The incubation of the peptide with $ONOO^-$ and 100 μM GSH revealed a $ONOO^-$ concentration-dependent formation of an adduct at 2300.10 Da corresponding to a Δm of 305.09 Da

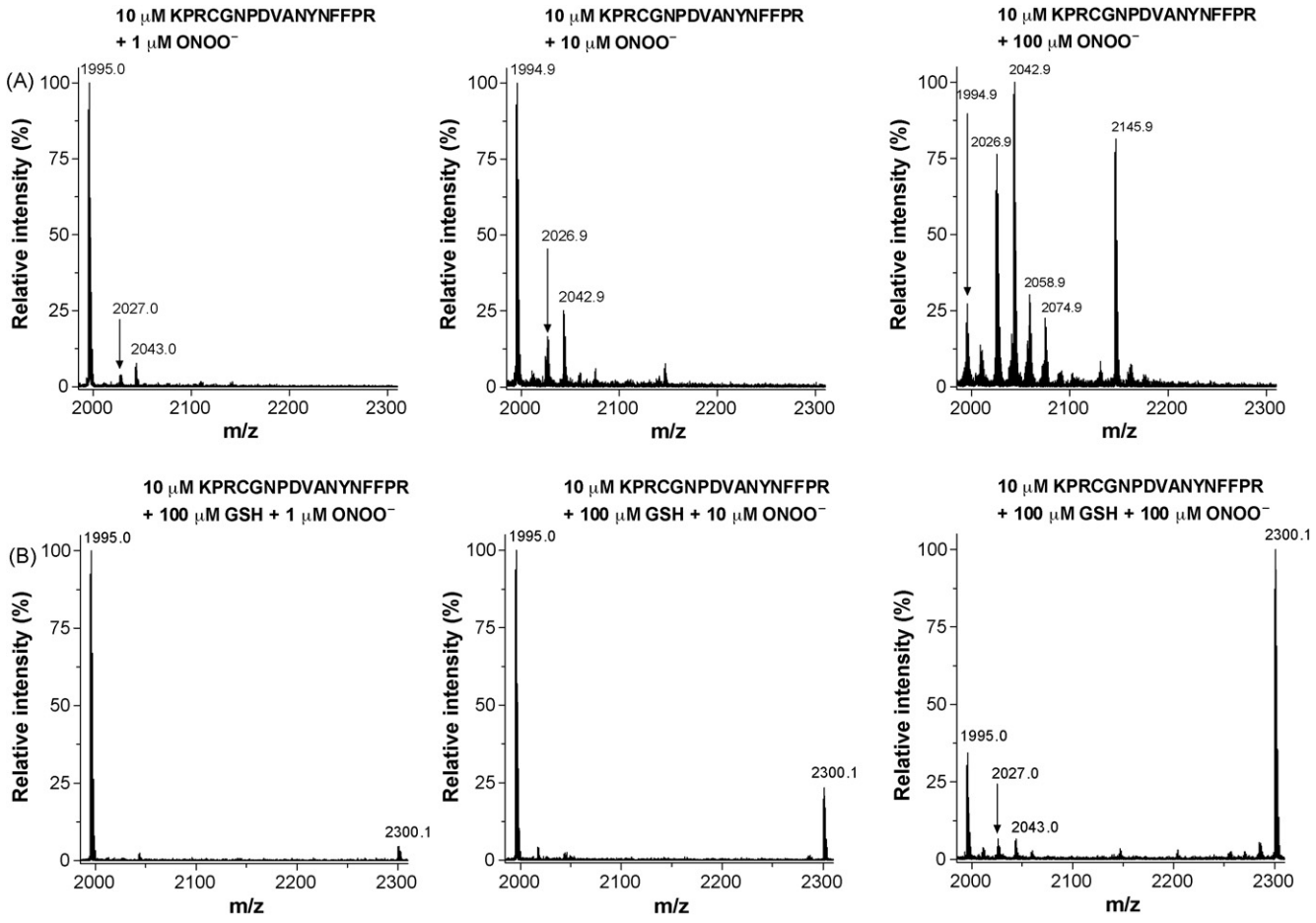

Fig. 3 – MALDI-TOF mass spectra of $ONOO^-$-induced modification of the synthetic fragment KPRCGNPDVANYNFFPR in the absence or presence of 100 μM GSH. 10 μM peptide was incubated with 1, 10 or 100 μM $ONOO^-$ in the absence (A) or presence (B) of 100 μM GSH in 50 mM Tris pH 8.2 for 15 min at 37 °C.

Table 1 – Modifications of the synthetic peptide KPRCGNPDVANYNFFR by $ONOO^-$ as identified by LC-MS/MS.

Treatment	*m/z*	z	Modified peptide
1 μM $ONOO^-$ pH 8.2	676.33	3	KPRC(diOx)GNPDVANYNFFPR
10 μM $ONOO^-$ pH 8.2	821.86	2	KPRC(triOx)GNPDVANYNF
100 μM $ONOO^-$ pH 8.2	821.88	2	KPRC(triOx)GNPDVANYNF
	681.66	3	KPRC(triOx)GNPDVANYNFFPR
	691.32	2	KPRC(triOx)GNPDVANY
	686.99	3	KPRC(triOx)GNPDVANY(Ox)NFFPR
1 μM $ONOO^-$ pH 7.6	676.32	3	KPRC(diOx)GNPDVANYNFFPR
	821.87	2	KPRC(triOx)GNPDVANYNF
10 μM $ONOO^-$ pH 7.6	691.32	2	KPRC(triOx)GNPDVANY
	681.66	3	KPRC(triOx)GNPDVANYNFFPR
	676.33	3	KPRC(diOx)GNPDVANYNFFPR
100 μM $ONOO^-$ pH 7.6	821.85	2	KPRC(triOx)GNPDVANYNF
	681.64	3	KPRC(triOx)GNPDVANYNFFPR
	676.31	3	KPRC(diOx)GNPDVANYNFFPR
	691.31	2	KPRC(triOx)GNPDVANY

Reaction mixtures were analyzed by LC-ESI-QTOF and MS/MS experimental data were searched against SwissProt database using Peaks Studio 4.5 and Mascot.

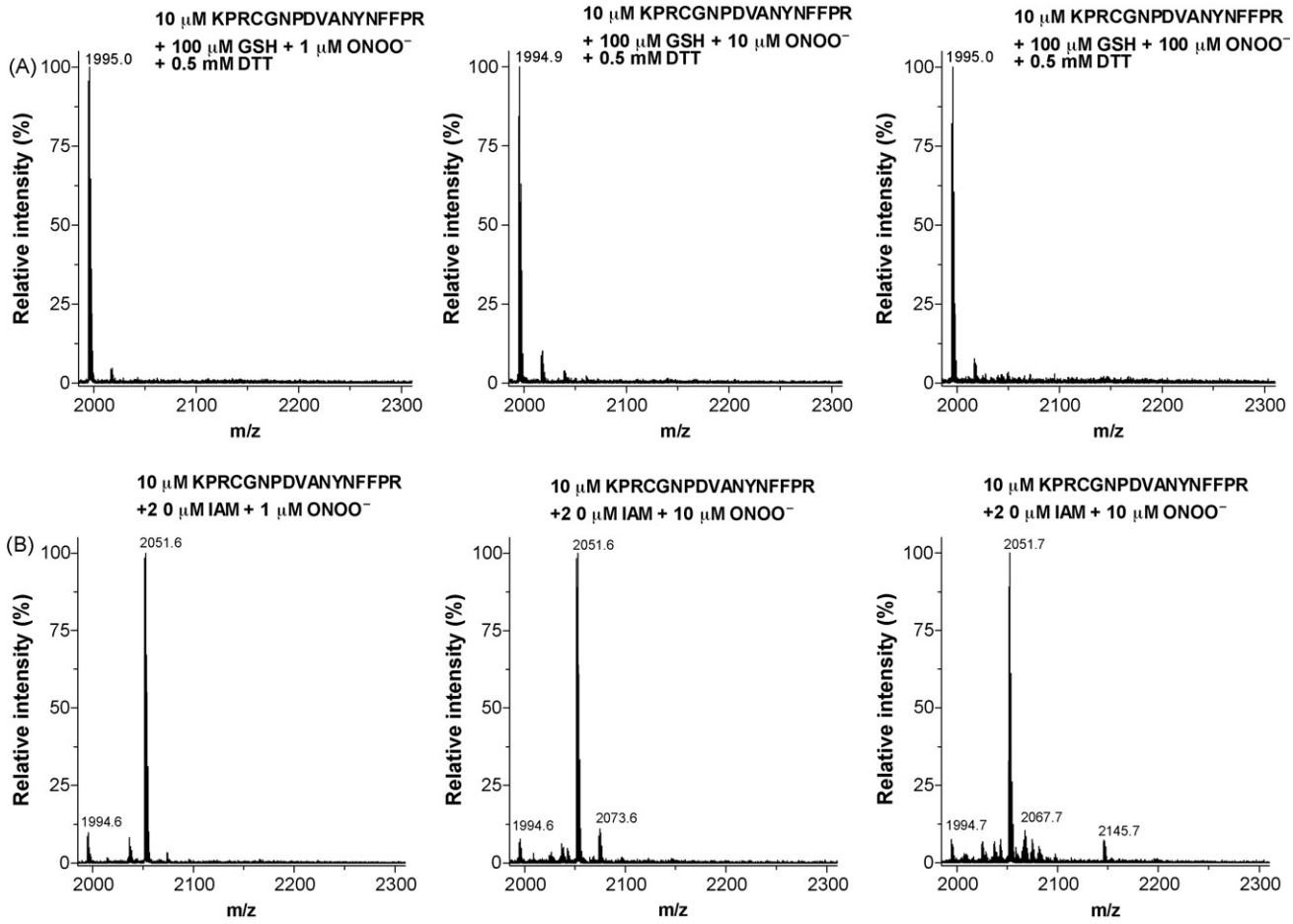

Fig. 4 – MALDI-TOF mass spectra of $ONOO^-$-induced modification of the synthetic fragment KPRCGNPDVANYNFFPR in the presence of GSH and dithiothreitol (DTT) (A) or iodoacetamide (IAM) (B). 10 μM peptide was incubated with 1 μM, 10 μM or 100 μM $ONOO^-$ in 50 mM Tris pH 8.2 containing 100 μM GSH for 15 min at 37 °C followed by the addition of 0.5 mM DTT for 30 min at 37 °C (A), or 20 μM IAM for 30 min at room temperature prior to incubation with 1 μM, 10 μM or 100 μM $ONOO^-$ in 50 mM Tris pH 8.2 for 15 min at 37 °C (B).

(Fig. 3B), likely representing an S-glutathiolated adduct (theoretical mass = 305.07 Da) at the Cys residue. Subsequent treatment of the reaction mixture with excess dithiothreitol (which reduces disulfides to thiols) resulted in the elimination of the glutathiolated adduct (Fig. 4A), suggesting that it is a reversible modification. Comparable results were obtained with 10 μM GSH, although more oxidation products than with 100 μM GSH were observed (Fig. S5). The incubation of the peptide with 20 μM iodoacetamide (which specifically alkylates Cys) prior to the addition of $ONOO^-$ resulted mainly in the formation of a product at 2051.60 Da corresponding to the addition of a carbamidomethyl group (Δm = 57.00 Da) (Fig. 4B).

MALDI-TOF spectra from samples obtained after in-solution tryptic digestion of 72 kDa MMP-2 revealed at least 10 signature masses (Fig. 5A), including the propeptide domain fragment CGNPDVANYNFFPR at 1613.57 Da, that were virtually identical (<0.1% variation) to those predicted by in silico trypsin digestion of human 72 kDa MMP-2. The incubation of 72 kDa MMP-2 with 10 μM or 100 μM $ONOO^-$ revealed an additional peak at 1661.58 Da, consistent with an oxidation of the propeptide domain fragment to a sulfonic acid derivative. Enzyme incubation with 10 or 100 μM $ONOO^-$ and 10 μM or 100 μM GSH resulted in a peak at 1918.59 Da (Fig. 5B), corresponding to an S-glutathiolated adduct of the CGNPDVANYNFFPR fragment (Δm = 305.02 Da).

4. Discussion

This work describes for the first time the modulation of MMP-2 activity by $ONOO^-$ and GSH, and possible modification of the Cys residue in the autoinhibitory domain of MMP-2 that may be involved in the enzyme's activation. This modulation resulted in a bell-shaped activation of the enzyme with low μM concentrations of $ONOO^-$ followed by its inactivation at higher $ONOO^-$ concentrations. Post-translational modification of 72 kDa MMP-2 by oxidative stress has been postulated to be a key event leading to its intracellular activation in various pathological conditions [4,5]. The activation of MMP-2 by $ONOO^-$ concentrations higher than 100 μM has been reported previously [18,19]. However, activation of the enzyme was only considered by the appearance of 65 kDa or lower gelatinolytic

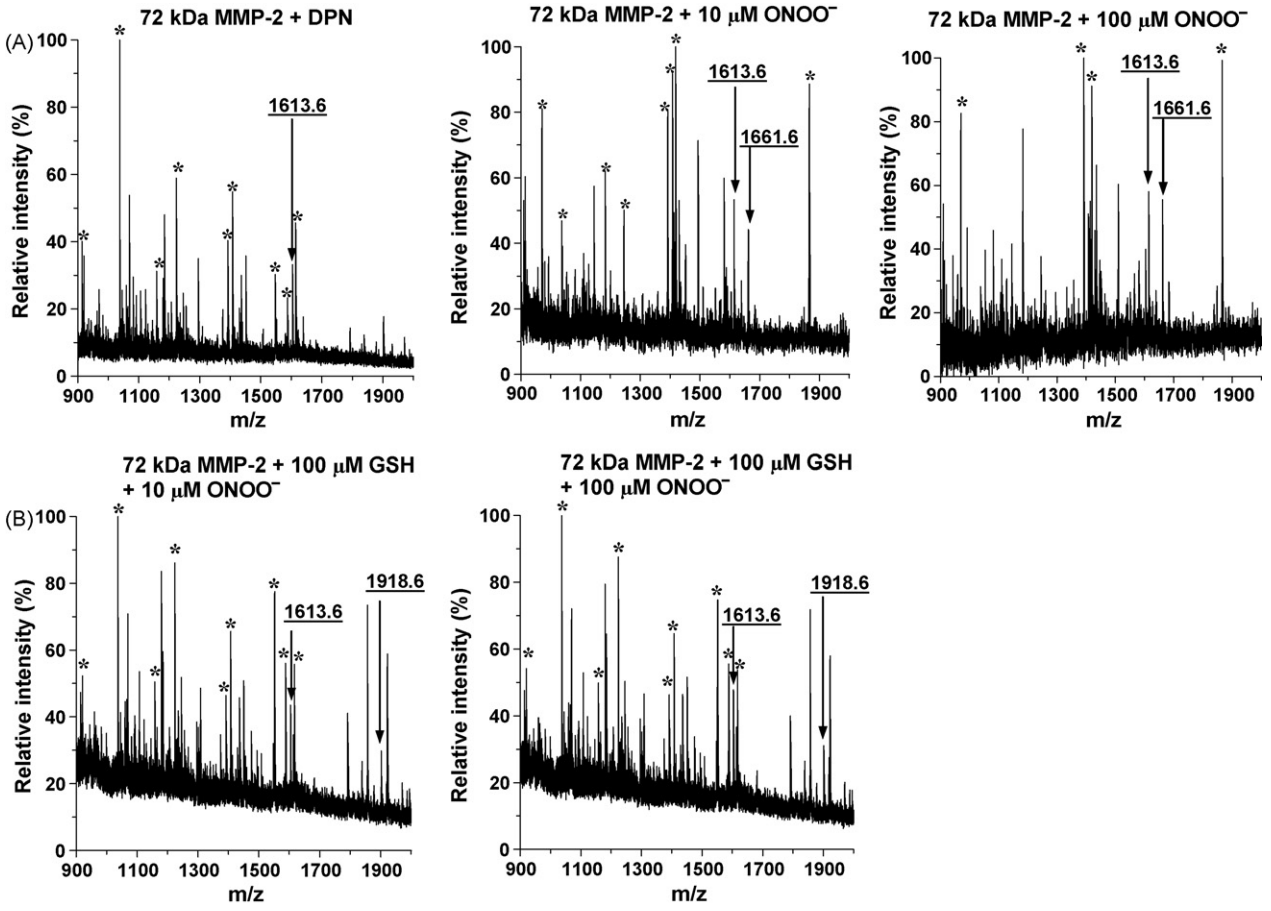

Fig. 5 – Peptide mass fingerprinting analysis of the modified cysteine residue in the autoinhibitory domain of human recombinant 72 kDa MMP-2. MALDI-TOF mass spectra of in-solution tryptic digest of 72 kDa MMP-2 (0.1 μM) treated with decomposed $ONOO^-$ (DPN) or $ONOO^-$ (10 or 100 μM) in the absence (A) or presence of 10 or 100 μM GSH (B) in 50 mM Tris pH 7.6 for 30 min at 37 °C. The masses for the unmodified (1613.6 Da), modified by oxidation (1661.6 Da) or S-glutathiolation (1918.6 Da) tryptic fragment CGNPDVANYNFFPR containing the Cys residue in the autoinhibitory domain are indicated. The asterisks indicate the signature masses of the kDa MMP-2 fragments.

activities in zymography, as it was hypothesized that the 72 kDa enzyme is an inactive zymogen and only the cleaved forms of the enzyme are active.

We observed that $ONOO^-$ can modulate the activity of 72 kDa MMP-2 with an increase in enzyme activity at 0.3–1 μM $ONOO^-$, and a loss of enzymatic activity at $ONOO^-$ concentrations exceeding 10 μM (Figs. 1 and 2). Furthermore, when OmniMMP® was used as a substrate, the effect of $ONOO^-$ was significant on the enzyme's V_{max}, but not on K_M. The apparent differences in optimal $ONOO^-$ concentrations required to achieve maximal MMP-2 activation in different assays may be due to the fact that kinetic parameters for different substrates would be expected to be influenced differently by $ONOO^-$, and the net change in measured initial velocities at subsaturating substrate concentrations would not necessarily parallel increases in V_{max}. Our mass spectrometry data suggest that $ONOO^-$-induced enzyme activation may involve the modification of the conserved Cys residue in the autoinhibitory domain which keeps the enzyme in a latent form. These data are consistent with the postulated cysteine-switch model of MMP activation [20].

The reaction of $ONOO^-$ with the sulfhydryl group in either free Cys, bovine serum albumin or GSH at pH 7.4 and 37 °C is rapid and ranges between 1.35 and $4.5 \times 10^3\ M^{-1}\ s^{-1}$ [21,22]. GSH has been reported to form a thionitrate ($GSNO_2$) when treated with bolus $ONOO^-$ [23]. $GSNO_2$ was found to fully activate the MMP-9 zymogen and to produce the glutathiolation of the synthetic peptide PRCVPD (containing the cysteine-switch in the autoinhibitory domain of the MMP-9 zymogen) via a disulfide S-oxide bond [11]. Although reasonable intermediates, thionitrate esters are not stable compounds and rapidly hydrolyse to yield sulfinyl and sulfonyl products, and in the presence of thiols they rapidly decompose to yield disulfides and inorganic nitrite [24]. Notably, at higher $ONOO^-$ concentrations we also observed tyrosine hydroxylation, but no nitration (Fig. 5). Tyrosine hydroxylation has been detected in $ONOO^-$-treated bovine serum albumin digests and hydrolysates from glial cells exposed to IL-1β (a cytokine that stimulates concomitant cellular production of $ONOO^-$) [25].

Reversible, regulatory post-translational modifications affecting Cys residues of various proteins (e.g., glycogen phosphorylase, *N*-methyl-D-aspartate glutamate receptor, α-

and β-tubulin, β- and γ-actin, ryanodine receptor) were observed under physiological conditions in the central nervous and cardiovascular systems with either S-nitrosothiols [15,26] or 3-morpholinosydnonimine [27], a $ONOO^-$-donor. Reversible modifications (i.e., S-nitrosylation and S-glutathiolation) can also occur in response to low concentrations of $ONOO^-$ (10–50 μM), such as the S-glutathiolation and subsequent activation of the sarco/endoplasmic reticulum calcium ATPase-2, an essential enzyme for the physiological regulation of vascular tone [13]. However, in pathological conditions (e.g., atherosclerosis), where higher $ONOO^-$ concentrations cause profound modifications of the protein structure and loss of catalytic activity, this enzyme undergoes more severe oxidative damage due to irreversible oxidation of critical Cys residues.

In our hands, GSH, previously shown to maximally activate MMP-1, -8 and -9 at equimolar $ONOO^-$ concentrations [11], attenuated the effect of $ONOO^-$ on the activity of 72 kDa MMP-2. This can reflect the scavenging of $ONOO^-$ by excess GSH [2,28]. Alternatively, GSH could exert an inhibitory effect on MMP-2 activity [28,29], or it may reversibly activate the inactivated enzyme, counteracting the effect of $ONOO^-$. Notably, equimolar concentrations of GSH promoted $ONOO^-$-mediated S-glutathiolation of the critical Cys residue in the autoinhibitory domain of 72 kDa MMP-2 as well as that in the synthetic peptide KPRCGNPDVANYNFFPR.

The comparison of mass spectrometry data for the reaction of PRCGVPD fragment of MMP-1, -8 and -9 with $ONOO^-$ [11] with our mass spectrometry data suggests that the product profile for the reaction of $ONOO^-$ with the MMP zymogen depends on the relative molar ratio of $ONOO^-$ to enzyme, and, when GSH is present, also on the molar ratio of $ONOO^-$ to GSH at any given time. Additionally, amino acid composition (there is less than 60% identity among 72 kDa MMP-2 and other MMP zymogens) and structural conformation of MMPs may affect the outcome of their interaction with $ONOO^-$.

Our observations suggest that the 72 kDa MMP-2 zymogen can be activated by $ONOO^-$ and that a subtle and complex balance between the relative intracellular levels of $ONOO^-$ and important redox active molecules such as GSH may occur. Conditions such as ischemia-reperfusion or proinflammatory cytokine-induced injury of the heart are associated with enhanced oxidative stress, in particular via $ONOO^-$ overproduction, MMP-2 activation and loss of contractile function [2,3,12,16,30], and may be understood, at least in part, in terms of $ONOO^-$-dependent activation of 72 kDa MMP-2 zymogen.

Acknowledgments

We thank the Institute of Biomolecular Design and Department of Chemistry, University of Alberta for access to their mass spectrometers. We thank Dr. Takeshi Adachi (Keio University, Tokyo, Japan) for scientific advice, and Jing Zheng, Paul Semchuk and Owen Degenhardt for technical assistance. This project was supported by the Canadian Institutes of Health Research Grants MOP-77526 to R.S. and MOP-77529 to A.H. R.S. is an Alberta Heritage Foundation for Medical Research Scientist. A.C.N. is a recipient of Alberta Heritage Foundation for Medical Research and Heart and Stroke Foundation of Canada Fellowships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.11.004.

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