THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 31, Issue of August 3, pp. 29596–29602, 2001
Printed in U.S.A.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Activation of Matrix Metalloproteinases by Peroxynitrite-induced Protein *S*-Glutathiolation via Disulfide *S*-Oxide Formation*

Received for publication, March 19, 2001, and in revised form, May 3, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M102417200

Tatsuya Okamoto‡§, Takaaki Akaike‡¶, Tomohiro Sawa‡, Yoichi Miyamoto‡, Albert van der Vliet§, and Hiroshi Maeda‡

From the ‡Department of Microbiology, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan and the §Division of Pulmonary/Critical Care Medicine, Department of Internal Medicine, School of Medicine, University of California, Davis, California 95616

Oxidative stress may cause tissue injury through activation of the precursors of matrix metalloproteinase (proMMPs). In this study, we observed glutathione (GSH)-dependent proMMP activation induced by peroxynitrite, a potent oxidizing agent formed during inflammatory processes. Peroxynitrite strongly activated all three types of purified human proMMPs (proMMP-1, -8, and -9) in the presence of similar concentrations of GSH. Of the potential reaction products between peroxynitrite and GSH, only *S*-nitroglutathione ($GSNO_2$) caused proMMP activation. Extensive *S*-glutathiolation of the proMMP protein occurred during activation of proMMP by peroxynitrite and GSH, as shown by radiolabeling studies with [^{35}S]GSH or [^{3}H]GSH. Evidence of appreciable *S*-glutathiolation persisted even after dithiothreitol and protein-denaturing treatment, however, suggesting that some *S*-glutathiolation did not occur through formation of simple mixed disulfide. Matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry indicated that not only peroxynitrite plus GSH but also synthetic $GSNO_2$ produced dithiothreitol-resistant *S*-glutathiolation of the synthetic peptide PRCGVPD, which is a well conserved Cys-containing sequence of the propeptide autoinhibitory domain of proMMPs. PRCGVPD *S*-glutathiolation is presumed to be formed through glutathione disulfide *S*-oxide (GS(O)SR), based on the *m/z* 1064. Our results illustrate a unique mechanism of oxidative proMMP activation and oxidative tissue injury during inflammation.

Extracellular matrix (ECM)[1] is known to be modulated by a number of matrix metalloproteinases (MMPs), which are critical for disintegration and remodeling of ECM under physiological and pathological conditions, *e.g.* wound healing, rheumatoid arthritis, periodontal disease, idiopathic pulmonary fibrosis (IPF), and tumor growth and invasion or metastasis (1–6). MMPs, a group of zinc neutral endopeptidases, are produced by a variety of cells and are released extracellularly as inactive precursors (proMMPs) (7–9). Activation of proMMPs can be achieved either by limited proteolysis of the zymogens or by chemical modification, such as by organomercurial compounds and reactive oxygen species (7, 8, 10). We previously found that some bacterial proteinases, particularly thermolysin family enzymes such as *Pseudomonas* elastase and *Vibrio cholerae* proteinase, can activate proMMPs via limited proteolysis of the autoinhibitory domain (11). Moreover, we showed that reactive nitrogen intermediates such as nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) activate human neutrophil proMMP (proMMP-8), which may be involved in neutrophil-induced tissue injury (12).

Peroxynitrite is a strong oxidizing and nitrating agent, which is formed from nitric oxide (NO) and superoxide anion radical (O_2^-) via a diffusion-limited rapid reaction (13, 14). Excessive production of peroxynitrite has been reported to cause oxidative and nitrative stress under various pathological conditions (14–18). For example, oxidative stress during inflammatory responses is often associated with tissue destruction (19), which may occur by a mechanism that depends on peroxynitrite-induced ECM disintegration.

Accumulated evidence shows that various antioxidants, including sulfhydryl-containing compounds (*e.g.* glutathione, GSH) (20), uric acid (21, 22), and selenium-dependent GSH peroxidase (23), can protect against peroxynitrite-induced cytotoxicity. GSH, a tripeptide (γ-Glu-Cys-Gly), is a sulfhydryl-containing compound with wide distribution in various organisms (24–26). This ubiquitous tripeptide is the most abundant intracellular thiol in cells; it regulates gene expression, cell proliferation and apoptosis, signal transduction, enzyme and protein functions, and oxidative stress in biological systems (26–28). In addition, protein *S*-glutathiolation through mixed disulfide formation is suggested to have physiological and pathophysiological consequences occurring during oxidative stress (29–33). The biological relevance of protein *S*-glutathiolation is, however, not fully clarified, particularly in oxidative stress-related pathology.

The intracellular concentration of GSH is maintained at a high level (1–8 mM) in a variety of cells, but in the extracellular milieu it exists at a low micromolar level. High levels of GSH (about 100 μM) are found in alveolar lining fluid in normal human subjects, and the GSH concentration is decreased in many inflammatory lung diseases including IPF and acute respiratory distress syndrome (28, 34–36). Our recent study indicated that different isoforms of MMPs are critically in-

This is an open access article under the CC BY license.

* This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) and the Ministry of Health, Labor and Welfare (Kouseiroudousho) of Japan (to T. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Microbiology, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan. Tel.: 81-96-373-5100; Fax: 81-96-362-8362; E-mail: takakaik@gpo.kumamoto-u.ac.jp.

[1] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; proMMP, precursors of MMP; IPF, idiopathic pulmonary fibrosis; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; NAC, *N*-acetyl L-cysteine; BSA, bovine serum albumin; GSNO, *S*-nitrosoglutathione; $GSNO_2$, *S*-nitroglutathione; MALDI-TOF MS, matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol.

This paper is available on line at http://www.jbc.org

volved in ECM remodeling of lung tissue in IPF (4), which seems to undergo oxidative stress as evidenced by reduced levels of GSH (36). Thus, it is of great importance to explore the role of GSH in the pathogenesis of oxidative stress-induced tissue damage as related to regulation of MMP activities, which may be affected by free radical species and reactive oxygen and nitrogen oxide intermediates, such as peroxynitrite, generated during inflammation.

In the present study, to obtain a better understanding of the regulatory function of GSH in ECM destruction induced by oxidative stress, we investigated the effect of GSH on peroxynitrite-mediated activation of proMMPs by using three types of purified human proMMPs (proMMP-1, -8, and -9). Our results revealed a unique mechanism of proMMP activation caused by *S*-glutathiolation of proMMPs, in which the GSH adduct of proMMP may be produced through disulfide *S*-oxide formation involving generation of *S*-nitroglutathione ($GSNO_2$) by peroxynitrite.

EXPERIMENTAL PROCEDURES

Substances—Human buffy coats were kindly supplied by Kumamoto Red Cross Blood Center, Kumamoto, Japan. The reduced form of glutathione (GSH; M_r 307.3), the oxidized form of glutathione (GSSG), *N*-acetyl L-cysteine (NAC), L-cysteine, acid-soluble human placenta type I collagen, and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma. α-Gelatin monomer was a product of Serva Feinbiochemica, GmbH, Heidelberg, Germany. Crystalline bovine serum albumin (BSA) was from Calbiochem, La Jolla, CA. *N*-Ethylmaleimide (NEM) was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. *Pseudomonas aeruginosa* elastase was obtained from Nagase Biochemicals, Osaka, Japan. The heptapeptide Pro-Arg-Cys-Gly-Val-Pro-Asp (PRCGVPD; M_r 742.8), which is a highly conserved motif in the propeptide domain of proMMP, was synthesized and provided by Bio-Factory, Asahi Techno Glass Corp., Funabashi, Japan. *S*-Nitrosoglutathione (GSNO) was supplied from Dojindo Laboratories, Kumamoto, Japan.

$GSNO_2$ was prepared and purified according to the method of Balazy *et al.* (37). Briefly, GSH was nitrated by reaction with NO_2BF_4 (Aldrich) at a molar ratio 1:4 (GSH:NO_2BF_4) in 10 mM phosphate-buffered saline (pH 7.4) for 10 min at room temperature. $GSNO_2$ thus formed was purified by reverse phase high performance liquid chromatography (HPLC; TSKgel ODS-80Ts, Tosoh Co., Ltd., Tokyo, Japan); elution was with 0.01% acetic acid plus 3% acetonitrile, and the fraction containing $GSNO_2$ was collected and lyophilized.

Peroxynitrite was synthesized by use of a quenched flow reactor as described previously (12). [^{3}H]GSH ([*glycine*-2-^{3}H]GSH; specific activity, 1658 GBq/mmol) and [^{35}S]GSH (γ-Glu-[^{35}S]Cys-Gly; specific activity, 7696 GBq/mmol) were obtained from Moravek Biochemicals, Brea, CA, and PerkinElmer Life Sciences, respectively. Because these radiolabeled GSH preparations contained dithiothreitol and GSH-derived impurities (GSSG or other GSH derivatives), we further purified the radiolabeled GSH compounds by HPLC (TSKgel ODS-4PW, Tosoh, mobile phase, 20 mM potassium phosphate buffer, pH 8.4, plus 100 μM diethylentriaminepentaacetic acid) before use in the present study. All other chemicals were of the highest analytical grade commercially available.

Purification of Human ProMMPs—Human neutrophil procollagenase (proMMP-8) and 92-kDa progelatinase (type IV procollagenase; proMMP-9) were purified from human neutrophils isolated from buffy coats by using series column chromatography (11). Purified human interstitial procollagenase (proMMP-1) from HT1080 cells, a human fibrosarcoma cell line, was a gift from Kanebo Biomedical Laboratory, Kanagawa, Japan (11). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed proMMP-1, -8, and -9 as single bands with apparent molecular sizes of 52, 85, and 92 kDa, respectively. Both proMMP-1 and -8 showed little or very low, if any, collagenolytic activity against human placenta type I collagen before activation. However, strong collagenolysis was produced by treatment with 1 mM PCMB, a well known activator of proMMPs. Similarly, proMMP-9 exerted strong gelatinolytic activity only after activation with PCMB. These findings indicate that the three types of proMMPs that we used are latent forms of proenzymes (11, 12).

ProMMP Activation after Treatment with Peroxynitrite and Various Thiol Compounds—The three different purified proMMPs (proMMP-1, -8, and -9; final concentration of each: 2 μM) were incubated with various concentrations of peroxynitrite in the presence or absence of GSH or with free thiols such as L-cysteine and NAC in 100 mM Tris-HCl buffer (pH 8.2) at 35 °C for 30 min, followed by incubation with their substrates (6 μg of type I collagen for MMP-1 and -8; 6 μg of gelatin for MMP-9) at 35 °C for 90 min. Because peroxynitrite has a short half-time at pH 8.2 ($t_{1/2}$ of 4.2 s), it was added three times at 5-min intervals. The reaction was stopped by boiling the reaction mixture with the treatment buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 40 mM dithiothreitol, pH 6.8) for 3 min, after which SDS-PAGE (7.5% acrylamide gel) was performed. After electrophoresis, the protein bands were stained with Coomassie Brilliant Blue R-250 (Quick CBB, Wako). Collagenolytic or gelatinolytic activities generated after the treatment were assessed by measuring the amount of the substrates degraded. The amounts were determined by densitometric analysis of SDS-PAGE gel on a Macintosh computer (Macintosh G3; Apple Computer Co., Ltd., Cupertino, CA) combined with an image scanner (GT6500 ART2; Epson Co., Ltd., Tokyo, Japan) using the public domain NIH Image software. Native type I collagen is a heterotetramer of α1(I) and α2(I) collagen monomers, and its specific digestion to the 3/4-length (α1A, α2A) and 1/4-length (α1B, α2B) fragments is brought about by MMP-1 and -8. In contrast, MMP-9 digestion of gelatin produces small molecular weight peptides. The activities of MMPs were assessed by measuring the percentage of digested substrates *versus* intact substrates. The activating potentials of GSSG, GSNO, and $GSNO_2$ for proMMPs were tested similarly.

Detection of S-Glutathiolation of the Autoinhibitory Domain of ProMMP—proMMP-9 (2 μM) was incubated with peroxynitrite (10 μM) and either [^{35}S]GSH or [^{3}H]GSH (0.4 μM each) in 100 mM Tris-HCl buffer, pH 8.2, at 35 °C for 30 min. To an aliquot of each reaction mixture was added an equal volume of the sample buffer (125 mM Tris-HCl buffer, pH 6.8, plus 4% SDS) with or without 80 mM dithiothreitol, followed by boiling of the mixture at 100 °C for 3 min and then electrophoresis on 10% SDS-PAGE. After electrophoresis, the gels were stained with Quick CBB (Wako) and dried, and the radioactive band on the gels was detected by using a bioimage analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan). In some experiments, before the radiolabeling reaction with GSH and peroxynitrite, proMMP-9 was treated with 2 mM PCMB or 2 mM NEM to modify the free sulfhydryls of the protein. Additionally, proMMP-9 treated with [^{35}S]GSH and peroxynitrite was digested at 35 °C for 90 min by *Pseudomonas* elastase (200 nM), which is known to cleave proMMP-9 at a specific region between the autoinhibitory and the active enzyme domains (11).

Similar autoradiographic analysis was performed with a sulfhydryl-containing BSA, after treatment with peroxynitrite in the presence of [^{35}S]GSH. Briefly, BSA (5 μM) was incubated with peroxynitrite (10 μM) and [^{35}S]GSH (0.4 μM) in 100 mM Tris-HCl buffer (pH 8.2) at 35 °C for 30 min, and the radiolabeling of the protein with [^{35}S]GSH was examined as just described.

Detection of GSH Adducts with Synthetic Peptide (PRCGVPD) by Matrix-assisted Laser-desorption Ionization-Time-of-flight Mass Spectrometry (MALDI-TOF MS)—Synthetic peptide (PRCGVPD; molecular mass, 742.8) (5 mM) was incubated with GSH (10 mM), GSH plus peroxynitrite (50 μM each), or $GSNO_2$ (25 μM) in 100 mM Tris-HCl, pH 8.2, at 35 °C for 30 min. Each solution was treated with or without dithiothreitol (20 mM) at 35 °C for 30 min and was analyzed by MALDI-TOF MS. A 0.5-μl aliquot of the reaction mixture was deposited and dried on a stainless steel probe tip, being overlaid with 0.5 μl of a matrix consisting of 10 mg/ml sinapinic acid (3, 5-dimethoxy-4-hydroxycinnanic acid, Aldrich) in 0.05% trifluoroacetic acid plus 50% acetonitrile. Molecular mass values of peptide derivatives were determined by means of MALDI-TOF MS (Shimadzu/Kratos Kompact MALDI III, Shimadzu Co., Kyoto, Japan). Conditions used for MALDI-TOF MS measurements were as follows: flight path, linear mode; polarity, positive; laser power, 75–95 eV. Molecular mass values were calibrated by using bovine insulin (M_r 5733.5; Sigma) as a standard molecule.

RESULTS

Activation of ProMMPs Induced by Peroxynitrite with or without GSH—As demonstrated in Fig. 1*A*, treatment of proMMP-8 with peroxynitrite resulted in a moderate and concentration-dependent activation, which is consistent with our earlier report (12). GSH strongly potentiated this peroxynitrite-induced proMMP-8 activation (Fig. 1*A*). The maximum activation of proMMP-8 was produced with peroxynitrite when it was added to the reaction mixture containing an equivalent concentration of GSH. The increase in proMMP-8 activation by

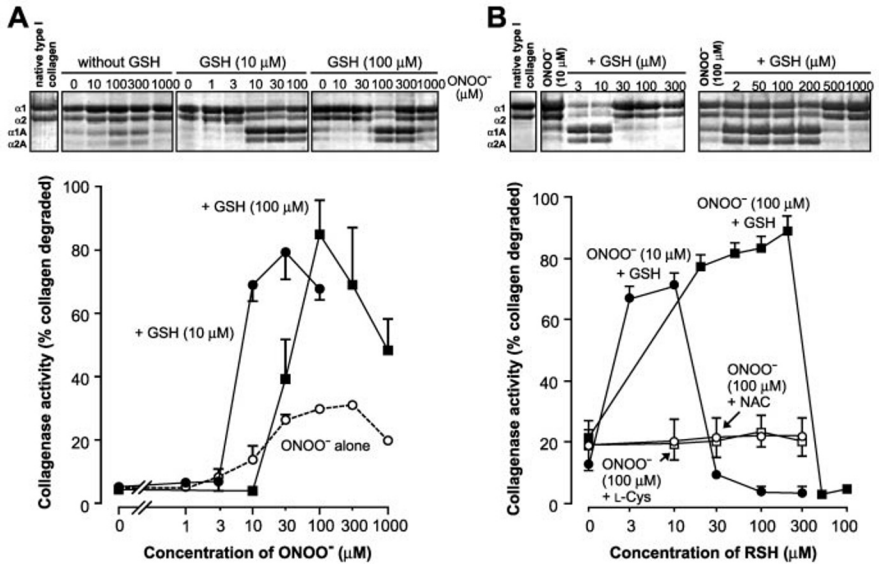

FIG. 1. **Activation of proMMP-8 by peroxynitrite and GSH.** After treatment of purified proMMP-8 (2 μM) with various concentrations of peroxynitrite ($ONOO^-$) with GSH (10, 100 μM) or without GSH in 100 mM Tris-HCl buffer, pH 8.2, at 35 °C for 30 min, the reaction mixtures were incubated with type I collagen (6 μg) at 35 °C for 90 min. SDS-PAGE of native type I collagen is shown as a protein-loading control on the *left end* of the *upper panels*. Collagenase activities generated were assessed by densitometric analysis of SDS-PAGE gel. Data are means ± S.D. of three experiments. See "Experimental Procedures" for details.

GSH was reduced slightly with excessive concentrations of peroxynitrite compared with GSH concentrations.

When the concentration of GSH was varied during treatment of proMMP-8 with a constant concentration of peroxynitrite, a dose-dependent generation of MMP-8 activity was observed as the GSH concentration increased and approached the peroxynitrite concentration (Fig. 1*B*). However, an excessive amount of GSH relative to the peroxynitrite concentration completely suppressed proMMP-8 activation. GSH, even at a high concentration (1000 μM), did not attenuate the activity of an active form of MMP-8 that was generated by *Pseudomonas* elastase via specific proteolytic processing and removal of the autoinhibitory domain of the zymogen, as we described previously (data not shown) (11). Therefore, almost complete inhibition of proMMP-8 activation by GSH appears not to be due to blocking of the Zn^{2+} ion in the active site via a metal-chelating effect. In addition, because other sulfhydryl-containing compounds such as L-cysteine and NAC did not affect peroxynitrite-mediated activation of proMMP-8 (Fig. 1*B*), thiol-potentiated proMMP-8 activation by peroxynitrite may be unique to GSH.

Although peroxynitrite alone did not appreciable activate proMMP-1 and -9, in the presence of GSH it did produce a remarkable activation of both proMMP-1 and -9 (Fig. 2), in the same manner as for proMMP-8. No significant activation of all proMMPs tested was observed with GSH (10–100 μM) in the absence of peroxynitrite. Additionally, neither collagenolysis nor gelatinolysis was observed without addition of proMMPs to the reaction mixture of peroxynitrite plus GSH (data not shown), indicating that collagen and gelatin degradations shown in Figs. 1 and 2 were solely dependent on MMP activities produced by GSH/peroxynitrite. Therefore, GSH seems to potentiate peroxynitrite-induced activation of proMMPs through a mechanism commonly operative during the reaction of peroxynitrite plus GSH with proMMPs.

Effect of Various GSH Derivatives on ProMMP-8 Activation—To test the possibility that proMMP-8 activation was achieved by some reaction product of GSH and peroxynitrite, the effect of GSSG, GSNO, and $GSNO_2$ on proMMP-8 activation was investigated. Treatment of proMMP-8 (2 μM) with GSH and peroxynitrite at equal concentrations of each compound (1, 10, and 100 μM) markedly activated the zymogen in a dose-dependent manner. GSNO and GSSG (1, 10, and 100 μM), however, had little activating potential for proMMP-8 (Fig. 3*A*). $GSNO_2$, which has been identified as a product of the reaction of GSH with peroxynitrite (37), caused strong activation of proMMP-8; linear and dose-dependent generation of MMP-8 activity was observed, as illustrated in Fig. 3*B*. These data suggest that formation of simple mixed disulfide by GSSG or GSNO and transnitrosylation from GSNO are not involved in proMMP activation, even if these derivatives are produced during peroxynitrite reactions. Rather, a nitration product of GSH ($GSNO_2$) may contribute to the peroxynitrite-dependent proMMP activation.

GSH Conjugation (S-Glutathiolation) to the ProMMP-9 Autoinhibitory Domain—The molecular interaction between proMMPs and GSH during proMMP activation was examined by radiochemical analysis of GSH-adduct formation of proMMP-9 produced by peroxynitrite. As shown in the *upper panel* of Fig. 4*A*, SDS-PAGE of proMMP-9 under reducing conditions showed a single and homogeneous 92-kDa band, but under nonreducing conditions three bands of 210, 150, and 92 kDa were produced. These three bands are attributable to the proMMP-9 dimer (210 kDa), the proMMP-9-lipocalin complex (150 kDa), and the proMMP-9 monomer (92 kDa). It should be noted that the molecular size of proMMP-9 assessed by SDS-PAGE did not change during treatment with peroxynitrite and GSH (Fig. 4*A*, *middle section* in *upper panel*), suggesting that proteolytic processing of the zymogen does not occur during peroxynitrite- and GSH-induced proMMP activation.

When proMMP-9 was treated with [^{35}S]GSH together with peroxynitrite, a strong radioactive band for the proMMP-9 proteins was evident on SDS-PAGE (Fig. 4*A*, *middle section* in the *lower panel*). Appreciable radiolabeling remained on SDS-PAGE even after the radiolabeled proteins were treated with dithiothreitol and were denatured by heating (100 °C, 3 min) in the presence of SDS. Urea treatment (1.27 M; 100 °C, 3 min) together with SDS and dithiothreitol was also ineffective at completely dissociating the [^{35}S]GSH binding with proMMP-9 produced by peroxynitrite (data not shown). These results indicate that GSH reacted with proMMP-9 at least in part via a bond distinct from a simple mixed disulfide. In contrast, addition of [^{35}S]GSH alone, without peroxynitrite, resulted in radiolabeling of the proMMP protein, which was completely reversed with dithiothreitol treatment under reducing conditions (Fig. 4*A*, *left* in the *lower panel*). Because proMMP-9 was not conjugated with [^{35}S]GSH after blocking of the sulfhydryls of proMMP-9 with use of the thiol modification agents PCMB and NEM (Fig. 4*A*, *right* in the *lower panel*), radiolabeling of proMMP-9 with [^{35}S]GSH is thought to be produced by *S*-glutathiolation induced by peroxynitrite. Similar results were

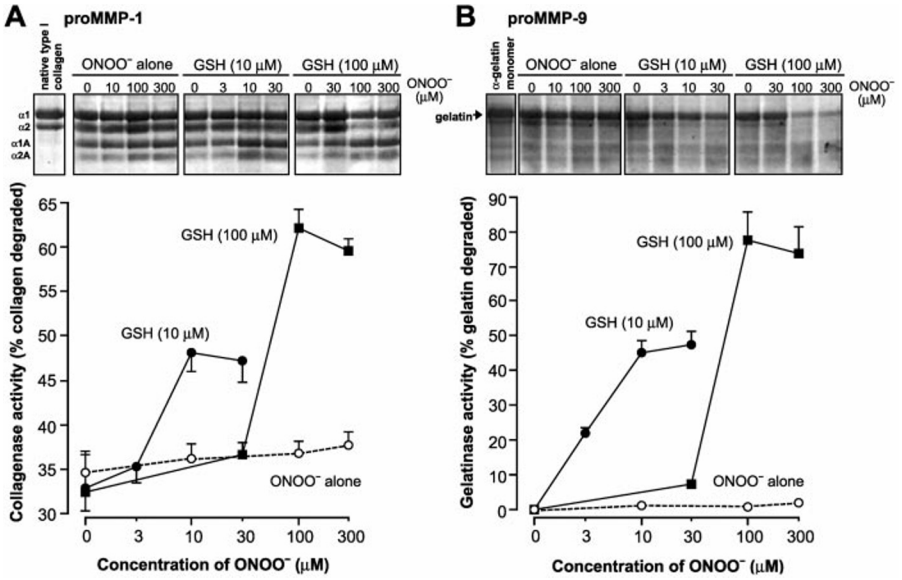

FIG. 2. **Activation of proMMP-1 and -9 by peroxynitrite and GSH.** After treatment of purified proMMP-1 (2 μM) (*A*) and proMMP-9 (2 μM) (*B*) with various concentrations of peroxynitrite with or without GSH (10 or 100 μM) in 100 mM Tris-HCl buffer, pH 8.2, at 35 °C for 30 min, the reaction mixtures were reacted with 6 μg of their substrates at 35 °C for 90 min. SDS-PAGE of native type I collagen or α-gelatin monomer is shown as a protein-loading control on the left end of the upper panel. Enzyme activities generated were assessed by the densitometric analysis of SDS-PAGE gel. Data are means ± S.D. of three experiments. See "Experimental Procedures" for details.

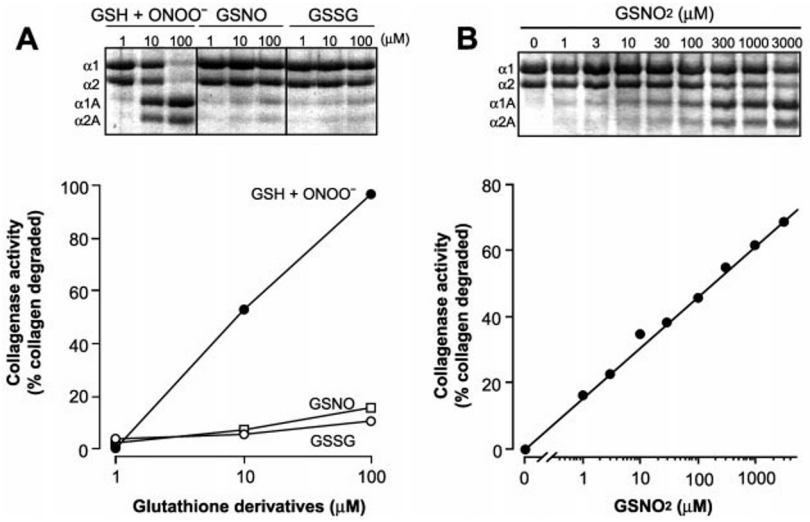

FIG. 3. **Effect of various GSH derivatives on activation of proMMP-8.** ProMMP-8 (2 μM) was treated with equal concentrations of GSH and peroxynitrite (1, 10, and 100 μM) or with GSNO or GSSG (1, 10, and 100 μM) without peroxynitrite (*A*), and collagenase activity generated was measured as described in Fig. 1. Similarly, proMMP-8 was incubated with various concentrations of $GSNO_2$, followed by measurement of its collagenolytic activity (*B*). Data are mean values of three experiments. See "Experimental Procedures" for details.

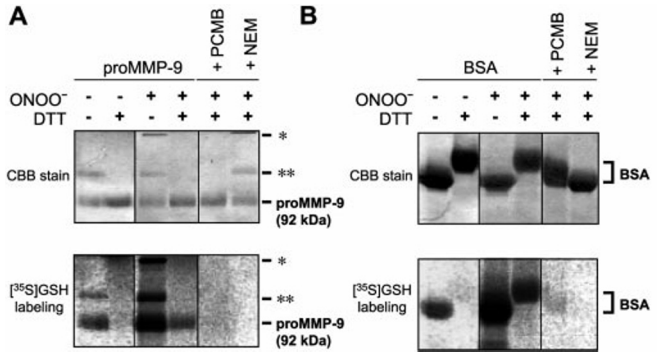

FIG. 4. ***S*-Glutathiolation of proMMP-9 (*A*) and BSA (*B*) determined by using radiolabeled GSH.** ProMMP-9 (2 μM) or BSA (5 μM) treated with [^{35}S]GSH (0.4 μM) in the presence or absence of 10 μM peroxynitrite was subjected to SDS-PAGE (10% acrylamide gel) under reducing or nonreducing conditions (with or without dithiothreitol (DTT)), and the protein band was stained with CBB. *Upper panels* show CBB staining of the protein bands; *lower panels*, radioactive bands as detected by a bioimage analyzer. The *asterisks* indicate the proMMP-9 dimer (*, 210 kDa) and the proMMP-9-lipocalin complex (**, 150 kDa). See "Experimental Procedures" for details.

observed with [^{3}H]GSH (data not shown). Following *Pseudomonas* elastase treatment of peroxynitrite/GSH-activated proMMP-9, which removes the autoinhibitory domain of proMMP-9 and generating an 82-kDa active form of MMP-9 (11), very little radioactivity of the major protein band of the proteinase-activated MMP-9 (82 kDa) was detected. These results suggest that GSH is coupled solely with the autoinhibitory domain of proMMP-9, not with the mature domain of the enzyme.

To examine whether *S*-glutathiolation induced by peroxynitrite occurs with sulfhydryls of other proteins, BSA treated with [^{35}S]GSH in the presence or absence of peroxynitrite was subjected to SDS-PAGE and autoradiographic analysis, similar to the studies with proMMP-9. As shown in Fig. 4*B*, a radioactive band, which was detected with BSA treated with [^{35}S]GSH without peroxynitrite, completely disappeared after treatment with dithiothreitol, indicating that the [^{35}S]GSH binding to BSA was through a disulfide bridging. In contrast, treatment of the protein with [^{35}S]GSH plus peroxynitrite greatly increased the intensity of the radioactive band, with a substantial level of radioactivity due to [^{35}S]GSH labeling remaining even after dithiothreitol treatment (Fig. 4*B*, *middle section* in the *lower panel*). Sulfhydryl modification of BSA totally blocked labeling with [^{35}S]GSH (Fig. 4*B*, *right section* in the *lower panel*). These data imply again that peroxynitrite may produce *S*-glutathiolation of various sulfhydryl-containing proteins via a covalent binding that seems to be distinct from a simple disulfide formation, as evidenced by different susceptibility to dithiothreitol treatment.

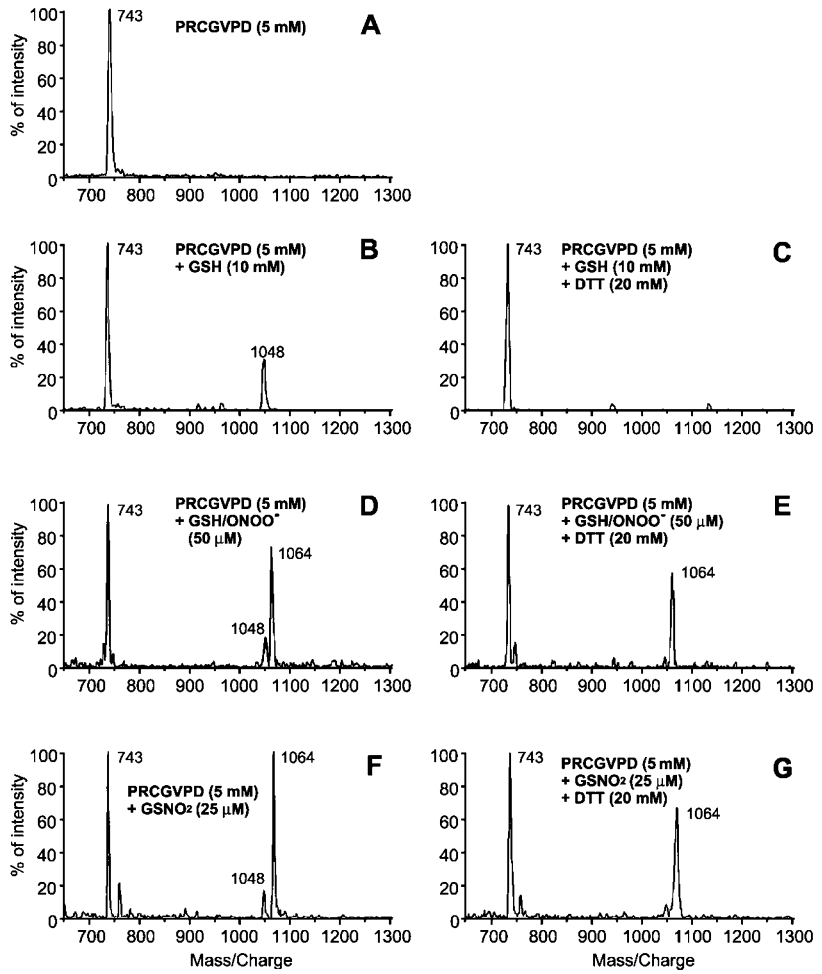

FIG. 5. **Peroxynitrite-induced *S*-glutathiolation of PRCGVPD through disulfide *S*-oxide formation as determined by MALDI-TOF MS.** The synthetic peptide PRCGVPD treated with GSH, with $GSNO_2$, or with GSH plus peroxynitrite with or without dithiothreitol was analyzed by MALDI-TOF MS. *A*, PRCGVPD alone; *B*, PRCGVPD plus GSH; *C*, as in *B*, plus DTT; *D*, PRCGVPD treated with GSH and peroxynitrite; *E*, as in *D*, plus DTT; *F*, PRCGVPD treated with $GSNO_2$; *G*, as in *F*, plus DTT. See "Experimental Procedures" for details.

Characterization of S-Glutathiolation of ProMMPs by MALDI-TOF MS—To further investigate the mechanism of proMMP activation caused by peroxynitrite/GSH or directly produced by $GSNO_2$, we used a synthetic peptide representing the conserved sequence of the proMMP autoinhibitory domain, PRCGVPD, and used MALDI-TOF MS to characterize the structure of the *S*-glutathiolated adduct of PRCGVPD produced by peroxynitrite/GSH or by $GSNO_2$. Chemical modification of the Cys residue in this sequence is essential for proMMP activation by chemical means without proteolytic processing of the zymogen. Analysis of PRCGVPD revealed a peak with m/z 743, as shown in Fig. 5*A*. Reaction of this peptide with GSH alone generated a product with m/z 1048 (Fig. 5*B*), which was completely eliminated by dithiothreitol (Fig. 5*C*). On the basis of the known masses of GSH (307 Da) and PRCGVPD (743 Da), this product is presumed to be a disulfide-bridged form of PRCGVPD plus GSH. The reaction of PRCGVPD with GSH/peroxynitrite produced another major component having m/z 1064, most of which remained even after dithiothreitol treatment; the simple glutathiolated adduct (m/z 1048) that was also generated, however, was eliminated by dithiothreitol (Fig. 5, *D* and *E*). The difference in mass between the two products is 16, which is identical with the mass of an oxygen atom. The same spectra were obtained following the reaction of PRCGVPD with $GSNO_2$ and dithiothreitol (Fig. 5, *F* and *G*), suggesting very similar chemical modification (*S*-glutathiolation) of PRCGVPD by peroxynitrite/GSH and $GSNO_2$. The present MS analysis suggests that the structure of the binding between GSH and the sulfhydryl of PRCGVPD was a mixed sulfinyl disulfide or glutathione disulfide *S*-oxide (GS(O)SR), which may contribute to potent proMMP activation induced by peroxynitrite plus GSH or by $GSNO_2$.

DISCUSSION

A number of MMPs play an important role in degradation and remodeling of ECM under physiological and pathological conditions (1–6). ProMMPs are produced by various cells, and their expression is regulated in different ways in each type of cell (7–10). For example, neutrophils upon stimulation release MMP-8 and -9 extracellularly from stored granules (12, 38). Many other cells such as fibroblasts, epithelial cells, macrophages, and tumor cells produce a diverse array of MMPs, including MMP-1, -2, and -9, stromelysin, matrilysin, and macrophage elastase (MMP-12). Some types of MMPs are upregulated by proinflammatory cytokines and growth factors such as tumor necrosis factor-α, interleukin-1β, and transforming growth factor-β1, leading to tissue injury in various inflammatory diseases (39, 40). All MMPs are produced as inactive precursors (proMMPs), and a specific activation process is a prerequisite of the generation of their proteolytic activity against ECM components (7, 8, 10, 11). ProMMPs consist of three discernible domains, referred to as the autoinhibitory domain, the zinc-binding catalytic domain, and the homopexin-like C-terminal domain (7, 9, 10). The sequence PRCGVPD in the autoinhibitory domain, which is highly conserved among all members of the proMMP family, is thought to be involved in maintaining the inactive form of the enzyme by coordinate binding of cysteine thiolate and zinc ion, which protects the active site from substrates. The key event during proMMP activation is dissociation of the binding of the thiolate moiety in

the PRCGVPD region and the zinc atom at the active center of the enzyme, leading to full accessibility of the enzyme to the ECM (*i.e.* the cysteine switch activation mechanism) (7, 10).

Various endogenous proteinases such as trypsin, chymotrypsin, plasmin, and cathepsin G have been implicated in proteolytic activation of proMMPs by virtue of their limited proteolysis of the autoinhibitory domain to produce mature forms of MMPs (7, 8, 10, 41). We previously found that proteolytic activation of proMMPs is also brought about quite effectively by some exogenous proteinases derived from pathogenic bacteria, which may contribute to the invasiveness and the pathogenic potential of such bacteria (11). Moreover, reactive oxygen species, particularly hypochlorite anion (OCl^-), are reported to activate proMMP-8, possibly through oxidative modification of the autoinhibitory domain, although the exact mechanisms and biological relevance of the oxidative activation of proMMPs remain unclear (38). As demonstrated in our previous work, similar activation of proMMP-8 purified from human neutrophils is induced by physiologically relevant micromolar concentrations of reactive nitrogen oxides such as NO_2 and peroxynitrite (12). However, the regulatory mechanism of proMMP activation occurring under oxidative stress has been poorly understood.

In this context, our present study revealed a unique mechanism of proMMP activation that is induced by peroxynitrite and GSH, possibly through *S*-glutathiolation of the autoinhibitory domain of proMMPs via formation of a stable disulfide *S*-oxide. This result suggests an important role of peroxynitrite as a potential mediator of oxidative tissue injury through MMP-dependent ECM destruction. This idea is further supported by our recent study showing involvement of peroxynitrite-dependent proMMP activation in enhanced vascular permeability in the skin of mice and guinea pigs and in an experimental murine solid tumor *in vivo* (42).

GSH, ubiquitously distributed in biological systems, is known to be an important molecule in defense against oxidative stress (26–28). GSH scavenges active oxygen species and peroxynitrite, and GSH is thus often decreased during oxidative stress occurring in various diseases. For example, although relatively high concentration of GSH (~100 μM) exists in the alveolar spaces of the lung (28, 34), the GSH concentration in the alveolar spaces is significantly decreased (less than 100 μM) in various inflammatory lung diseases including acute respiratory distress syndrome and IPF (28, 35, 36); plasma in normal human subjects contains only low micromolar levels of GSH. MMP activity appears to be regulated by GSH in a manner that is based on a delicate redox balance of GSH affected by peroxynitrite. Specifically, under physiological conditions, a high concentration of GSH suppresses the activation of proMMPs and protects the ECM against disintegration induced by peroxynitrite-mediated oxidative stress. Prolonged and sustained inflammation, however, will produce a high level of proMMP expression in the inflammatory foci along with excessive amounts of reactive oxygen and nitrogen intermediate species such as superoxide, NO, and their product peroxynitrite. Excessive oxidant production thus causes a reduction of local concentrations of GSH, as just mentioned. Accordingly, oxidative stress will tip the balance between GSH and peroxynitrite in favor of oxidative glutathiolation of proMMP (at the autoinhibitory domain), leading to accelerated proMMP activation and ECM destruction. It is interesting that the tissue inhibitor of metalloproteinase-1, an endogenous MMP inhibitor, is readily inactivated by peroxynitrite (43), which suggests that peroxynitrite may potentiate MMP activity not only by direct activation of proMMPs but also by preservation of MMP activity after it is generated. Another important postulate related to glutathiolation-induced proMMP activation is that the thiolate ligand located in the well conserved motif PRCGVPD of the autoinhibitory domain of proMMPs may function as a redox sensor for the triggering of ECM disintegration in both physiological and pathological conditions.

In the present study, only GSH induced proMMP activation with the help of peroxynitrite; other sulfhydryl-containing compounds such as L-cysteine and NAC did not. This result suggests a unique function of GSH in the chemical modification of proMMPs (*S*-glutathiolation). Glutathiolation of proMMPs via disulfide *S*-oxide may drastically distort the structure and function of the autoinhibitory domain of proMMPs, possibly because of an as yet unidentified interaction with amino acid residues in the vicinity of the glutathiolation. In contrast, several reports have revealed a complicated picture of chemical reactions of sulfhydryls with NO and peroxynitrite (20, 37, 44–50). For example, various oxidized, nitrosylated, and nitrated derivatives of GSH are produced by peroxynitrite or NO. Of a series of these derivatives, $GSNO_2$ seems to be the most important molecular species involved in proMMP activation by *S*-glutathiolation through disulfide *S*-oxide formation, as we found in the present study.

Formation of $GSNO_2$ from the reaction of peroxynitrite and GSH has been described by Balazy *et al.* (37). Similar to their study, our MALDI-TOF MS analysis showed generation of $GSNO_2$ from GSH by peroxynitrite (data not shown). These results are further supported by the fact that both authentic $GSNO_2$ and $GSNO_2$ formed in the reaction of peroxynitrite and GSH exhibit the same potential for activating proMMP and forming a glutathione disulfide *S*-oxide adduct of PRCGVPD. $GSNO_2$ formation by peroxynitrite may be explained at least in part by a mechanism of tyrosine nitration by peroxynitrite, as proposed by Lymar's and Goldstein's groups (51, 52). They suggested that nitrotyrosine is formed via reaction of tyrosine with radicals $^{\bullet}OH$ and $^{\bullet}NO_2$ that have escaped from a solvent-caged radical pair of ONOOH ($ONO^{\bullet}$, $^{\bullet}OH$) (51). Thus, analogous to the tyrosine nitration reaction, glutathione thiyl radical ($GS^{\bullet}$) might first be formed by reaction of GSH with either $^{\bullet}OH$ or $^{\bullet}NO_2$, and then $GSNO_2$ could be produced by a coupling of the radicals $GS^{\bullet}$ and $^{\bullet}NO_2$. $GS^{\bullet}$ generation from GSH by peroxynitrite was clearly documented by Karoui *et al.* (20).

Another question remains, however. How does $GSNO_2$ participate in disulfide *S*-oxide formation in proteins and peptides? Thermodynamic analysis of organic thionitrate ($RSNO_2$) isomers has suggested that concerted rearrangement of thionitrate occurs, resulting in formation of isomers such as sulfenyl nitrite (RSONO) and sulfinyl nitrite (RS(O)NO), with subsequent homolytic generation of NO (53). A proposed mechanism for this NO generation is a homolysis-recombination pathway of thionitrate: $RSNO_2 \rightarrow (RS^{\bullet}, {}^{\bullet}NO_2) \rightarrow RSONO \rightarrow RSO^{\bullet} + {}^{\bullet}NO$. Indeed, NO release from $GSNO_2$ was demonstrated by Balazy *et al.* (37), suggesting indirectly that GSH sulfinyl radical ($GSO^{\bullet}$), which is the counterpart of sulfenyl nitrite and sulfinyl nitrite for NO, may be generated to yield disulfide *S*-oxide in the proteins.

Protein *S*-glutathiolation was also reported to be involved in oxidative regulation of various enzymatic and protein functions *in vitro* (29–33). Although a previous report described GSSG activating neutrophil procollagenase (proMMP-8) via disulfide-thiol exchange (simple mixed disulfide) (54), in our current study we did not obtain apparent proMMP activation by oxidized and nitrosated GSH derivatives, including GSSG and GSNO (Fig. 3*A*). Of considerable importance is a recent finding that effective *S*-glutathiolation is produced by glutathione disulfide *S*-oxide (GS(O)SG) that is formed during spontaneous decomposition of GSNO in aqueous solution (50). The glutathi-

one disulfide *S*-oxide is so reactive as to readily give rise to protein *S*-glutathiolation through a simple mixed disulfide. In contrast, protein and peptide (PRCGVPD) disulfide *S*-oxides seem to be substantially more stable than glutathione disulfide *S*-oxide, as evidenced by their resistance to dithiothreitol treatment, which we verified in our present work. In addition, GSNO formation has been suggested to occur during the reaction of peroxynitrite and GSH (48). Because GSNO had no effect on proMMP activation, glutathione disulfide *S*-oxide does not participate in any way in the unique *S*-glutathiolation of the proteins through disulfide *S*-oxide.

Although we did not identify the *S*-glutathiolation site in BSA, it has a free Cys residue at position 34 and its surrounding sequence ^{29}QYLQQCPFDEH^{39} (55), which is most likely to be modified by GSH/peroxynitrite. Only a few amino acids such as Pro, Asp, and some hydrophobic amino acids (Gly, Val, and Phe) commonly exist at downstream of the free Cys residue of BSA and the consensus PRCGVPD motif of proMMPs. A separate experiment showed that GSH/peroxynitrite produced much lower but appreciable level of *S*-glutathiolation of human α_1-proteinase inhibitor compared with *S*-glutathiolation of proMMP-9 and BSA (data not shown). α_1-Proteinase inhibitor has a single Cys at position 232 (56), and its Cys-containing sequence (^{227}FNIQHCKKLSS^{237}) is completely different from the PRCGVPD motif and the Cys-containing sequence in BSA. Therefore, there is little specificity, if any, in the amino acid sequence for the protein *S*-glutathiolation induced by GSH/peroxynitrite. It may be possible, however, that there is an as yet unidentified three-dimensional structure of polypeptides, which may determine the susceptibility to and stability of the disulfide *S*-oxide formation during *S*-glutathiolation. In any event, further study is needed to clarify the structural characteristics for protein *S*-glutathiolation via disulfide *S*-oxide formation.

In conclusion, a unique protein sulfhydryl modification, *i.e.* protein disulfide *S*-oxide formation, occurs during the reaction of peroxynitrite and GSH, possibly mediated by $GSNO_2$. ProMMP activation resulting from such sulfhydryl modification may be one of the important physiological and pathological consequences. Protein *S*-glutathiolation was evident not only with proMMPs but also with other sulfhydryl-containing proteins such as BSA. It is therefore conceivable that protein glutathione disulfide *S*-oxide is one of the post-translational modifications of sulfhydryl-containing polypeptides. Such modifications are likely generated during oxidative stress and contribute to various physiological and pathophysiological phenomena *in vivo*.

Acknowledgment—We thank Judith B. Gandy for excellent editorial work on our manuscript.

REFERENCES

1. Salo, T., Makela, M., Kylmaniemi, M., Autio-Harmainen, H., and Larjava, H. (1994) *Lab. Invest.* **70,** 176–182
2. Mainardi, C. L. (1985) *Ann. N. Y. Acad. Sci.* **460,** 345–354
3. Sorsa, T., Ding, Y., Salo, T., Lauhio, A., Teronen, O., Ingman, T., Ohtani, H., Andoh, N., Takeha, S., and Konttinen, Y. T. (1994) *Ann. N. Y. Acad. Sci.* **732,** 112–131
4. Suga, M., Iyonaga, K., Okamoto, T., Gushima, Y., Miyakawa, H., Akaike, T., and Ando, M. (2000) *Am. J. Respir. Crit. Care Med.* **162,** 1949–1956
5. Chambers, A. F., and Matrisian, L. M. (1997) *J. Natl. Cancer Inst.* **89,** 1260–1270
6. Westermarck, J., and Kahari, V. M. (1999) *FASEB J.* **13,** 781–792
7. Springman, E. B., Angleton, E. L., Birkedal-Hansen, H., and Van Wart, H. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 364–368
8. Grant, G. A., Goldberg, G. I., Wilhelm, S. M., He, C., and Eisen, A. Z. (1992) *Matrix* **1,** (suppl.) 217–223
9. Massova, I., Kotra, L. P., Fridman, R., and Mobashery, S. (1998) *FASEB J.* **12,** 1075–1095
10. Van Wart, H. E., and Birkedal-Hansen, H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5578–5582
11. Okamoto, T., Akaike, T., Suga, M., Tanase, S., Horie, H., Miyajima, S., Ando, M., Ichinose, Y., and Maeda, H. (1997) *J. Biol. Chem.* **272,** 6059–6066
12. Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M., Ichimori, K., and Maeda, H. (1997) *Arch. Biochem. Biophys.* **342,** 261–274
13. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1620–1624
14. Beckman, J. S., and Koppenol, W. H. (1996) *Am. J. Physiol.* **271,** C1424–C1437
15. Ischiropoulos, H. (1998) *Arch. Biochem. Biophys.* **356,** 1–11
16. Reiter, C. D., Teng, R. J., and Beckman, J. S. (2000) *J. Biol. Chem.* **275,** 32460–32466
17. Sawa, T., Akaike, T., and Maeda, H. (2000) *J. Biol. Chem.* **275,** 32467–32474
18. Akaike, T., and Maeda, H. (2000) *Immunology* **101,** 300–308
19. Weiss, S. J. (1989) *N. Engl. J. Med.* **320,** 365–376
20. Karoui, H., Hogg, N., Frejaville, C., Tordo, P., and Kalyanaraman, B. (1996) *J. Biol. Chem.* **271,** 6000–6009
21. Skinner, K. A., White, C. R., Patel, R., Tan, S., Barnes, S., Kirk, M., Darley-Usmar, V., and Parks, D. A. (1998) *J. Biol. Chem.* **273,** 24491–24497
22. Squadrito, G. L., Cueto, R., Splenser, A. E., Valavanidis, A., Zhang, H., Uppu, R. M., and Pryor, W. A. (2000) *Arch. Biochem. Biophys.* **376,** 333–337
23. Sies, H., Sharov, V. S., Klotz, L. O., and Briviba, K. (1997) *J. Biol. Chem.* **272,** 27812–27817
24. Meister, A., and Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52,** 711–760
25. Griffith, O. W. (1999) *Free Radic. Biol. Med.* **27,** 922–935
26. Sies, H. (1999) *Free Radic. Biol. Med.* **27,** 916–921
27. Meister, A. (1994) *Cancer Res.* **54,** 1969.s-1975s
28. Rahman, I., and MacNee, W. (2000) *Free Radic. Biol. Med.* **28,** 1405–1420
29. Ziegler, D. M. (1985) *Annu. Rev. Biochem.* **54,** 305–329
30. Thomas, J. A., Poland, B., and Honzatko, R. (1995) *Arch. Biochem. Biophys.* **319,** 1–9
31. Cotgreave, I. A., and Gerdes, R. G. (1998) *Biochem. Biophys. Res. Commun.* **242,** 1–9
32. Reddy, S., Jones, A. D., Cross, C. E., Wong, P. S., and van der Vliet, A. (2000) *Biochem. J.* **347,** 821–827
33. Davis, D. A., Dorsey, K., Wingfield, P. T., Stahl, S. J., Kaufman, J., Fales, H. M., and Levine, R. L. (1996) *Biochemistry* **35,** 2482–2488
34. van der Vliet, A., O'Neill, C. A., Cross, C. E., Koostra, J. M., Volz, W. G., Halliwell, B., and Louie, S. (1999) *Am. J. Physiol.* **276,** L289–L296
35. Pacht, E. R., Timerman, A. P., Lykens, M. G., and Merola, A. J. (1991) *Chest* **100,** 1397–1403
36. Cantin, A. M., Hubbard, R. C., and Crystal, R. G. (1989) *Am. Rev. Respir. Dis.* **139,** 370–372
37. Balazy, M., Kaminski, P. M., Mao, K., Tan, J., and Wolin, M. S. (1998) *J. Biol. Chem.* **273,** 32009–32015
38. Weiss, S. J., Peppin, G., Ortiz, X., Ragsdale, C., and Test, S. T. (1985) *Science* **227,** 747–749
39. Salo, T., Lyons, J. G., Rahemtulla, F., Birkedal-Hansen, H., and Larjava, H. (1991) *J. Biol. Chem.* **266,** 11436–11441
40. Ries, C., and Petrides, P. E. (1995) *Biol. Chem. Hoppe-Seyler* **376,** 345–355
41. Nagase, H., Enghild, J. J., Suzuki, K., and Salvesen, G. (1990) *Biochemistry* **29,** 5783–5789
42. Wu, J., Akaike, T., Hayashida, K., Okamoto, T., Okuyama, A., and Maeda, H. (2001) *Jpn. J. Cancer Res.* **92,** 439–451
43. Frears, E. R., Zhang, Z., Blake, D. R., O'Connell, J. P., and Winyard, P. G. (1996) *FEBS Lett.* **381,** 21–24
44. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *J. Biol. Chem.* **266,** 4244–4250
45. Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992) *Science* **258,** 1898–1902
46. Akaike, T. (2000) *Free Radic. Res.* **33,** 461–469
47. Hogg, N., Singh, R. J., and Kalyanaraman, B. (1996) *FEBS Lett.* **382,** 223–228
48. van der Vliet, A., 't Hoen, P. A., Wong, P. S., Bast, A., and Cross, C. E. (1998) *J. Biol. Chem.* **273,** 30255–30262
49. Viner, R. I., Williams, T. D., and Schoneich, C. (1999) *Biochemistry* **38,** 12408–12415
50. Li, J., Huang, F. L., and Huang, K. P. (2001) *J. Biol. Chem.* **276,** 3098–3105
51. Coddington, J. W., Hurst, J. K., and Lymar, S. V. (1999) *J. Am. Chem. Soc.* **121,** 2438–2443
52. Goldstein, S., Czapski, G., Lind, J., and Merenyi, G. (2000) *J. Biol. Chem.* **275,** 3031–3036
53. Cameron, D. R., Borrajo, A. M. P., Bennet, B. M., and Thatcher, G. R. J. (1995) *Can. J. Chem.* **73,** 1627–1638
54. Tschesche, H., and Macartney, H. W. (1981) *Eur. J. Biochem.* **120,** 183–190
55. Silvester, J. A., Timmins, G. S., and Davies, M. J. (1998) *Free Radic. Biol. Med.* **24,** 754–766
56. Miyamoto, Y., Akaike, T., Alam, M. S., Inoue, K., Hamamoto, T., Ikebe, N., Yoshitake, J., Okamoto, T., and Maeda, H. (2000) *Biochem. Biophys. Res. Commun.* **267,** 918–923