[Frontiers in Bioscience 7, a9-14, February 1, 2002]

PROTEIN DENITRATION/MODIFICATION BY *ESCHERICHIA COLI* NITRATE REDUCTASE AND MAMMALIAN CYTOCHROME P-450 REDUCTASE

Wu-Nan Kuo, Joseph M. Kocis and Jenny Webb (Kreahling)

Division of Science and Mathematics, Bethune-Cookman College, Daytona Beach,Florida 32114

TABLE OF CONTENTS

1. ABSTRACT

The incubation of peroxynitrite (PN)-pretreated histone III-S (NH) with *Escherichia coli* nitrate reductase (cytochrome, NADPH/GSH-independent) and that of NADPH-treated NH (NH_{NADPH}) with liver cytochrome P-450 reductase (NADPH-dependent) resulted in decreased 3-nitrotyrosine immunoreactivity found in Western blot analysis. Additionally, increased nitrate was noted as an end product of these reactions. These findings imply that varied enzymatic denitration/modification of NO/PN-reacted protein, either with or without a reductant, may be important in regulating related signal transduction cascade(s) and relieving oxidative stress.

2. INTRODUCTION

Cytochrome P-450 reductase is a NADPH-dependent flavoprotein, which is a component of the liver (and possibly other tissues) microcosmal mixed-function oxygenase system (1, 2). The transferring of electrons to cytochrome P-450 by the reductase is crucial in the detoxification of drugs and xenobiotics (1,3,4,2). Interestingly, cytochrome P-450 can catalyze the denitration of organic nitrates (5,6,3,7) and the denitrosation of nitrosoguanidinium compounds (8, 9). Such denitration/denitrosation of non-protein compounds may be important for cellular detoxification.

Via nonenzymatic reaction(s), tyrosine residues of proteins can be nitrated to form 3-nitrotyrosine by nitric oxide (NO) and/or its derivative peroxynitrite (PN) (10). 3-nitrotyrosine is a hallmark of NO/PN-mediated oxidative stress on proteins/enzymes, and it has been detected in the diseased tissues of Parkinson's (11), Alzheimer's (11, 12, 13), artherosclerotic lesions (11, 14), Huntington's (15, 12), multiple sclerosis (13), and autoimmune diseases (15). Additionally, PN can inactivate a variety of enzymes including α_1-antiproteinase (16), glutamine synthase (17), gylceraldehyde-3-phosphate dehydrogenase (18), acotinase (19), cytochrome P-450 (20), Ca^{2+}- ATPase of the sarcoplasmic reticulum (21), mitochondrial creatine kinase

(22), tyrosine hydroxylase (23), Mn^{2+}-superoxide dismutase (24), prostacyclin synthase (25), tissue inhibitor of metalloprotease-1 (26), glutathione reductase (27), nicotinamide nucleotide transhydrogenase (28), mitochondrial electron transport (29) and glutathione peroxidase (30). Surprisingly, modification by peroxynitrite can also lead to the activation of certain enzymes including cyclooxygenase (31), poly ADP synthetase (32) and matrix metalloprotease (33). Moreover, recent studies reveal the occurrence of enzymatic denitration/modification of NO/PN-reacted proteins (34, 35). However, further detailed investigations are required to elucidate the mechanism of these vital enzymatic reactions.

3. MATERIALS AND METHODS

3.1. Materials

Sodium dodecyl sulfate (SDS), NADPH, reduced glutathione (GSH), acrylamide, EGTA, EDTA, pepstatin-A, bestatin, leupeptin, transepoxysuccinyl-L-leucylamido(4-guanido)-butane, histone III-S (from calf thymus), bovine serum albumin (BSA), cytochrome P-450 reductase (from rabbit liver), and *Escherichia coli* nitrate reductase (cytochrome, NADPH/GSH-independent) were supplied by Sigma Chemical Company, St. Louis, USA. Western blotting reagents, nitrocellulose membranes, and anti-rabbit IgG alkaline phosphatase conjugate were obtained from Bio-Rad Laboratories, Hercules, USA. Peroxynitrite and rabbit polyclonal anti-nitrotyrosine were purchased from Upstate Biotechnology, Lake Placid, USA. Microcon centrifugal filter tubes (3,000 and 10,000Da-cut off) were from Millipore Corporation, Bedford, USA. Dialysis tubing (3,500Da-cut off) was purchased from Pierce, Rockford, USA. Nitrate/Nitrite Colorimetric Assay Kit was acquired through Cayman Chemical, Ann Arbor, USA.

3.2. Preparation of peroxynitrite (PN)-treated histone III-S (NH) and subsequent NADPH or GSH-treated NH (NH_{NADPH} or NH_{GSH})

Nine mg of histone III-S was treated with 1 mM PN at 25°C for 30 minutes in a final volume of 500µl of 50mM potassium phosphate buffer (pH 7.4) containing 1mM $FeCl_3$ and 1mM EDTA (36). Peroxynitrite (PN)-treated histone III-S (NH), thus prepared, was then dialyzed against 50 mM potassium phosphate buffer (pH 7.4) at 4°C for 6 hours to remove excess PN, $FeCl_3$ and EDTA. The dialyzed NH was further treated with 0.5 mM NADPH at 25°C for 15 minutes, and excessive NADPH was removed with centrifugal filter tubes (10kDa-cut off). The resulting samples (NH_{NADPH}) were dissolved in an appropriate volume of 50mM potassium phosphate buffer (pH 7.4) for the subsequent study with *Escherichia coli* nitrate reductase and cytochrome P-450 reductase. In addition, the further treatment of NH with 1 mM GSH (NH_{GSH}), the treatment of BSA with PN (NTB), and the subsequent treatment of NTB with NADPH (NTB_{NADPH}) or GSH (NTB_{GSH}) were similarly performed.

3.3 Enzymatic modification of NH, NH_{NADPH} and NH_{GSH}

With minor modifications, the experimental procedures basically followed those from past studies (34, 35, 37). The incubation mixture was in a final volume of 125 µl of 50 mM potassium buffer (pH 7.4) containing 75µg of NH, NH_{NADPH} or NH_{GSH}, 1mM of various protease inhibitors (pepstatin-A, bestatin, leupeptin, and transepoxysuccinyl-L-leucylamido(4-guanido)-butane) and varied amounts of modification enzyme (either *E. coli* nitrate reductase or cytochrome P-450 reductase). The reaction was carried out for 60 minutes at 30°C, and then terminated by the addition of 25µl of 10% SDS (without β-mercaptoethanol or diothiothreitol) followed by 30 seconds of boiling. The samples were subjected to SDS-polyacrylamide gel (12%) electrophoresis (SDS-PAGE) (38) and subsequent Western blot analysis. The detection of 3-nitrotyrosine was performed by using polyclonal rabbit anti-nitrotyrosine (0.05µg/ml) (38) as the primary antibody and anti-rabbit IgG-AP conjugate (39) as the secondary antibody. Finally, the enzymatic modification of treated NH, NH_{NADPH} and NH_{GSH} was monitored by their altered immunoreactivities.

3.4. Enzymatic denitration of NH, NH_{NADPH} and NH_{GSH}

The incubation mixture was in a final volume of 350µl of 50mM potassium phosphate buffer (pH 7.4) containing 450µg of NH, NH_{NADPH} or NH_{GSH}, 1mM of various protease inhibitors, and appropriate amount of enzyme. The reaction was carried out for 60 minutes at 30°C, and then terminated by boiling for 30 seconds followed by centrifugation at 16,000 x g at 4°C for 15 minutes to remove denatured proteins. The resulting supernatants were filtered through microcon centrifugal filter tubes (3kDa-cut off) at 16,000 x g at 4°C for 40 minutes and the filtrates were collected. Alternatively, the incubated samples were subject to centrifugal filtration without boiling. Finally, the concentrations of nitrate in the filtrates were determined by using the Nitrate/Nitrite Colorimetric Assay Kit (34, 35).

4. RESULTS AND DISCUSSION

To prevent the occurrence of an undesirable artifact, such as conversion of a nitro-group into an amino-group, the reducing agent (ß-mercaptoethanol or dithiothreitol) was omitted, and sample boiling was decreased from 60 seconds to 30 seconds prior to SDS-PAGE (37). As a result, the decrease in the intensity of immunoreactive bands was accurately assessed in Western blot.

The incubation of nitrated histone III-S (NH) with *E. coli* nitrate reductase (Figure 1, lane 2) drastically decreased the immunoreactivity as compared to the control sample, NH alone (Figure 1, lane 1). In contrast, the subsequent treatment of 1mM GSH (NH_{GSH}) (Figure 1, lane 3) did not significantly change the immunoreactivity of histone as compared to the control sample, NH alone (Figure 1, lane 1). Interestingly, striking similarity of decreased immunoreactive bands (Figure 1, lane 2) were noted when NH_{GSH} was incubated with *E. coli* nitrate reductase (Figure 1, lane 4). These results imply that 1mM GSH-treatment does not significantly alter the affinity between NH and the reductase, but rather, the enzymatic modification of NH is GSH-independent.

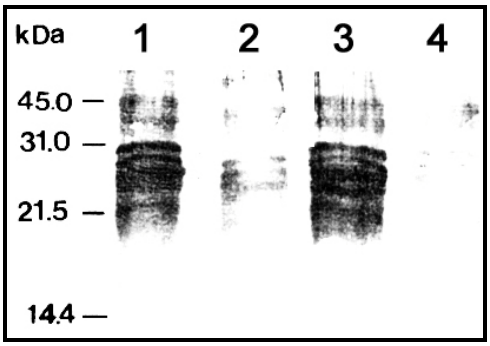

Figure 1. Modification of peroxynitrite-treated histone III-S (NH, 75µg) by *E. coli* nitrate reductase (5µg). Lane 1, NH alone; Lane 2, NH incubated with nitrate reductase; Lane 3, NH treated with 1mM GSH (NH_{GSH}); Lane 4, NH_{GSH} incubated with nitrate reductase.

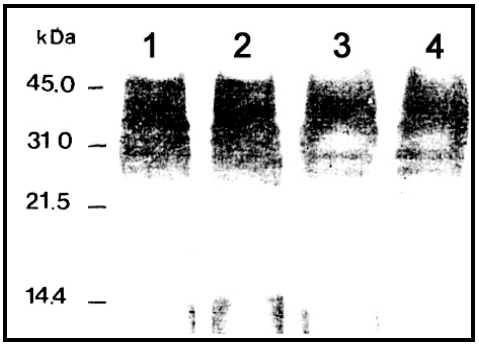

Figure 2. Lack of significant proteolysis on peroxynitrite-treated histone III-S (NH, 75µg) incubated with *E. coli* nitrate reductase (5µg). Incubation mixture contained various protease inhibitors and the resulting gel from SDS-PAGE (12%) was stained with Coomassie blue. Lane 1, NH alone; Lane 2, NH incubated with nitrate reductase; Lane 3, NH treated with 1mM GSH (NH_{GSH}); Lane 4, NH_{GSH} incubated with nitrate reductase.

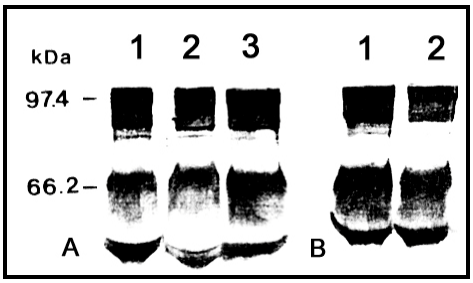

Figure 3. Lack of significant modification on peroxynitrite-treated BSA (NTB, 75µg) by *E. coli* nitrate reductase (5µg). (A) Lane 1, NTB alone; Lane 2, NTB incubated with nitrate reductase; Lane 3, NTB treated with 0.5mM NADPH (NTB_{NADPH}) prior to incubation with nitrate reductase. (B) Lane 1, NTB treated with 1mM GSH (NTB_{GSH}); Lane 2, NTB_{GSH} incubated with nitrate reductase.

Acquired through Sigma, histone III-S (from calf thymus), which is rich in lysine and primarily contains f_1 subgroup, has an approximate molecular mass of 22kDa (personal communication with Sigma). Nonetheless, the presence of other subgroups of histone and contaminant proteins has been suggested by the observation of multiple bands between 14.0 and 45.0kDa (Figure 1, lanes 1 and 3). This is further supported by the appearance of multiple bands of histone stained with Coomassie blue after SDS-PAGE (Figure 2).

Throughout the entire experiment, the possible interference due to protease contaminants in *E. coli* nitrate reductase was overcome by the addition of sufficient protease inhibitors in the incubation mixture. Regardless of incubation with or without the enzyme, the lack of apparent proteolysis was persistently noted in all samples by the unchanged intensity and mobility of peptide bands of NH and NH_{GSH} stained with Coomassie blue after SDS-PAGE (Figure 2). Hence, the modification of nitrated protein (histone III-S) without the production of smaller proteolytic fragments (Figure 2, lanes 2 and 4), indeed, is due to enzymatic modification of histone f_1 and contaminant proteins.

Subsequent treatment of PN-treated BSA (NTB) with 0.5mM NADPH (NTB_{NADPH}) or 1mM GSH (NTB_{GSH}) did not alter its immunoreactivity (data not shown). Additionally, the incubation of NTB (Figure 3A, lane 2), NTB_{NADPH} (Figure 3A, lane 3), and NTB_{GSH} (Figure 3B, lane 2) with *E. coli* nitrate reductase did not change the intensity/pattern of their immunoreactive bands as compared to control samples, NTB (Figure 3A, lane 1) and NTB_{GSH} alone (Figure 3B, lane 1). These results suggest that NTB, NTB_{NADPH} and NTB_{GSH} lack substrate specificity for *E. coli* nitrate reductase.

In this study, the pretreatment of 0.5mM NADPH may alter the 3-nitrotyrosine of NH as evident by a minor decrease in the immunoreactivity (Figure 4, lane 1). However, such treatment may facilitate rendering the substrate (NH_{NADPH}) to the active site of cytochrome P-450 reductase. This is supported by the drastically decreased immunoreactivity as the amount of the enzyme increases from 3, 5, to 10µg (Figure 4, lanes 2, 3, 4). In contrast, without NADPH-pretreatment to the substrate (NH) (Figure 4, lanes 5 and 6), no significant modification was observed as shown by the unchanged immunoreactivity (Figure 4, lane 6). These results suggest that the modification of NH by cytochrome P-450 reductase is NADPH-dependant.

The enzymatic modification of NH by *E. coli* nitrate reductase (GSH-independent) and cytochrome P-450 (NADPH-dependant) led to the production of nitrate, an end product, at approximately 1,300?mol and 3,000?mol, respectively (Table 1). In comparison, when BSA was substituted for *E. coli* nitrate reductase and cytochrome P-450 reductase as a control, there was no net nitrate produced (Table 1). The significant production of nitrate (Table 1), together with the decrease in immunoreactivity (Figure 1, lanes 2 and 4; Figure 4, lanes

Table 1. Enzymatic denitration of NH and NH_{NADPH}

Substrate (450mg)	Enzyme/Protein (10mg)	Net Nitrate Produced (rmol)
Experiment 1		
NH	BSA (control)	˜ 0
NH	Escherichia coli nitrate reductase	1,248 ± 98
Experiment. 2		
NH_{NADPH}	BSA (control)	˜ 0
NH_{NADPH}	Cytochrome P-450 reductase	2,976 ± 203

Enzymatic denitration was established under Materials and Methods. Each value shown is the mean ± S.E. from 3 to 5 samples. NH represents PN-treated histone III-S, and NH_{NADPH} represents NH further treated with 0.5mM NADPH.

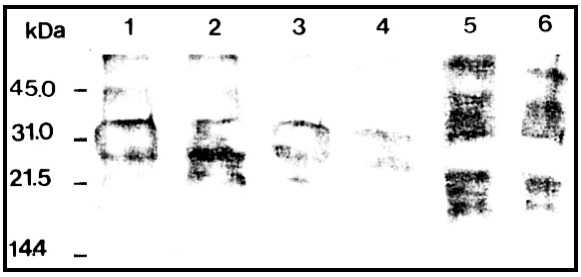

Figure 4. Modification of NH_{NADPH} (NH treated with 0.5mM NADPH, 75µg) by liver cytochrome P-450 reductase. Lane 1, NH treated with 0.5mM NADPH; Lanes 2-4, NH_{NADPH} incubated with 3, 5, and 10µg of cytochrome P-450 reductase, respectively; Lane 5, NH alone; Lane 6, NH incubated with 5µg of cytochrome P-450 reductase.

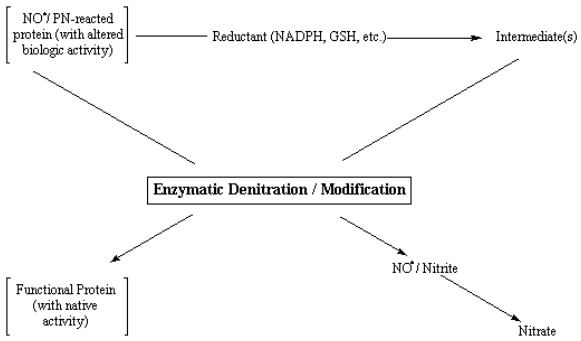

Figure 5 Scheme of enzymatic denitration/modification of NO/ PN-reacted proteins.

2-4) suggests the enzymatic modification of histone f_1 and contaminant proteins.

Enzymatic denitration/modification of NO/PN-reacted proteins is illustrated in Figure 5. Nitric oxide/peroxynitrite-reacted proteins, which may have significantly altered activities (34, 35), could form a complex or other intermediate(s) by further reaction with NADPH, thus, leading to favorable conformational change(s) for final enzymatic denitration/modification by cytochrome P-450 reductase. Moreover, other enzymes in cooperation with a reductant, such as GSH, might similarly modify NO/PN-reacted proteins. Alternatively, other enzymatic modification of these proteins may be reductant-independent, because it is possible that *E. coli* nitrate reductase-like enzymes also occur in the mammalian counterpart. These enzymatic modifications, which lead to the production of nitrate, may be due to the denitration of nitrotyrosine, nitrotryptophan and/or denitrosation of S-nitrosocysteine or N-nitroso amino acid (11). These enzymatic modifications could restore the biologic activities of proteins, thereby playing a vital role in regulating NO/PN-mediated signal transduction cascade(s) as well as relieving cellular oxidative stress.

5. ACKNOWLEDGEMENT

This work was supported by grant GM08119 from National Institutes of Health, USA.

6. REFERENCES

1. Backes WL: NADPH-Cytochrome P450 reductase. In: Cytochrome P450. Eds: Schenckman JB, Greim H, Springer-Verlag, Germany (1993)

2. Shephard EA, Pike SF, Rabin BR and Phillips IR: A rapid one-step purification of NADPH-cytochrome c(P-450) reductase from rat liver microsomes. *Anal Biochem* 129, 430-433 (1983)

3. McDonald BJ and Bennett BM: Cytochrome P-450 mediated biotransformation of organic nitrates. *Can J Physiol Pharmacol* 68, 1552-1557 (1990)

4. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA and Sligar SG: The catalytic pathway of cytochrome P450cam at atomic resolution. *Science* 287, 1615-1622 (2000)

5. Habig WH, Keen JH and Jakoby WB: Glutathione S-transferase in the formation of cyanide from organic thiocyanates and an organic reductase. *Biochem Biophys Res Commun* 64, 501-506 (1975)

6. Keen JH, Habig WH and Jakoby WB: Mechanism for the several activities of the glutathione S-transferase. *J. Biol Chem* 251, 6183-6188 (1976)

7. Schroder H: Cytochrome P-450 mediated bioactivation of organic nitrates. *Can J Physiol Pharmacol* 68, 298-302 (1992)

8. Jensen DE, Stelman GJ and Williams AW: Microsomally-mediated denitrosation of nitrosoguanidinium. *Carcinogenesis* 11, 1075-1082 (1990)

9. Jensen DE and Mackay RL: Rat, mouse and hamster isozyme specificity in the glutathione transferase-mediated denitrosation of nitrosoguanidinium compounds. *Cancer Research* 50, 1440-1448 (1990)

10. Balabanli B, Kamisaki Y, Martin E and Murad F: Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. *PNAS* 96, 13136-13141 (1997)

11. Berlett BS and Stadtman ER: Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272, 20313-20316 (1997)

12. Heales SJR, Bolanos JP, Stewart VC, Brookes PS, Land JM and Clark JB: Nitric oxide, mitochondria and neurological disease. *Biochim Biophys Acta* 1410, 215-28 (1999)

13. Smith MA, Richey Harris PL, Sayre LM, Beckman JS and Perry G: Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci*. 17, 2653-2657 (1997)

14. Bachmaier K, Nikolaus N, Pummerer C, Duncan GS, Mak TW, Matsuyama T, and Peninger J: iNOS expression and nitrotyrosine formation in the myocardium in response to inflammation is controlled by the interferon regulatory transcription factor 1. *Circulation* 96, 585-591 (1997)

15. Browne SE, Ferrante RJ and Beal MF: Oxidative stress in Huntington's disease brain. *Pathol* 9, 147-63 (1999)

16. Whiteman M and Halliwell B: Thiols and disulphides can aggravate peroxynitrite-dependent inactivation of alpha 1-antiproteinase. *FEBS Lett* 414, 497-500 (1997)

17. Berlett BS, Levine RL and Stadtman ER: Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase: both modifications mimic effects of adenylylation. *Proc. Natl Acad Sci USA* 95, 2784-9 (1997)

18. Souza JM and Radi R: Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. *Arch Biochem Biophys* 360, 187-94 (1998)

19. Gardner PR, Costantino, G, Szabo C and Salzman AL: Nitric oxide sensitivity of the aconitases. *J Biol Chem* 272, 25071-6 (1997)

20. Roberts ES, Lin HL, Crowley JR, Vuletich JL, Osawa Y and Hollenberg PF: Peroxynitrite-mediated nitration of tyrosine and inactivation of the catalytic activity of cytochrome P450 B1. *Chem Res Toxicol* 11, 1067-74 (1998)

21. Viner RI, Huhmer AF, Bigelow DJ and Schoneich C: The oxidative inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase by peroxynitrite. *Free Radic Res* 24, 243-59 (1996)

22. Stachowiak O, Dolder M, Walliman T and Richter C: Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J Biol Chem* 273, 16694-9 (1998)

23. Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J and Ischiropoulos H: Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci* USA 95, 7659-63 (1998)

24. Yamakura F, Taka H, Fujimura T and Murayama K: Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J Biol Chem* 273, 14085-14089 (1998)

25. Zou M, Martin C and Ullrich V: Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem* 378, 707-713 (1997)

26. Frears ER, Zhang Z, Blake DR, O'Connell JP and Winyard PG: Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Lett* 381, 21-4 (1996)

26. Francescutti D, Baldwin J, Lee L, and Mutus B: Peroxynitrite modification of glutathione reductase: modeling studies and kinetic evidence suggest the modification of tyrosines at the glutathione disulfide binding site. *Protein Eng* 9, 189-94 (1996)

28. Forsmark-Andree P, Persson B, Radi R, Dallner G and Ernster L: Oxidative modification of nicotinamide nucleotide transhydrogenase in submitochondrial particles effect of endogenous ubiquinol. *Arch Biochem Biophys* 336, 113-20 (1996)

29. Cassina A and Radi R: Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch Biochem Biophys* 328, 309-16 (1996)

30. Padmaja S, Squadrito GL and Pryor WA: Inactivation of glutathione peroxidase by peroxynitrite. *Arch Biochem Biophys* 349, 1-6 (1998)

31. Salvemini D: Regulation of cyclooxygenase enzymes by nitric oxide. *Cell Mol Life Sci* 53, 576-82 (1997)

32. Szabados E, Fischer GM, Toth K, Csete B, Nemeti B, Trombitas K, Habon T, Endrei D and Sumega B: Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of the AZT-induced cardiomyopathy in rat. *Free Radic Biol Med* 26, 309-17 (1999)

33. Maeda H, Tatsuya O and Akaike T: Human matrix metalloprotease activation by insults of bacterial infection involving proteases and free radicals. *Biol Chem* 379, 193-200 (1998)

34. Kuo WN, Kanadia RN and Shanbhag VP: Denitration of peroxynitrite-treated proteins by "protein nitratases" from dog prostate. *Biochem Mol Bio International* 47, 1061-1067 (1998)

35. Kuo WN, Kanadia RN, Shanbhag VP, and Toro R: Denitration of peroxynitrite-treated proteins by 'protein nitratases' from rat brain and heart. *Mol Cell Biochem* 201, 11-16 (1999)

36. Ye YZ, Strong M, Huang Z and Beckman JS: Antibodies that recognize nitrotyrosine. In: Methods in Enzymology. Ed: Packer L, Academic Press, NY (1996)

37. Kuo WN, Kreahling, JM, Shanbhag VP, Shanbhag PP and Mewar M: Protein nitration. *Mol Cell Biochem* 214, 121-9 (2000)

38. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227, 680-685 (1970)

39. Turner BM: The use of alkaline-phosphatase-conjugated second antibody for the visualization of electrophoretically separated proteins recognized by monoclonal antibodies. *J Immunol Meth* 63, 1-6 (1983)

Key Words: Nitric Oxide, Peroxynitrite, Protein Nitration/Denitration, Cytochrome P-450 Reductase, Nitrate Reductase (Cytochrome)

Send correspondence to: Dr. Wu-Nan Kuo, Division of Science and Mathematics, Bethune- Cookman College, Daytona Beach, Florida 32114, Tel: 386-481-2697, Fax: 386-481-2662, E-mail: kuow@cookman.edu