Tyrosine nitration site specificity identified by LC/MS in nitrite-modified collagen type IV

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Abbreviations: 3-NT, 3-nitrotyrosine; CID, collision induced dissociation; ECM, extra-cellular matrix; MPO, myeloperoxidase; NEG, non-enzymatic glycation; SRM, selected reaction monitoring

Abstract

Non-enzymatic nitrite induced collagen cross-linking results in changes reminiscent of age-related damage and parallels the well-known model system, non-enzymatic glycation. We have recently observed that nitrite modification of basement membrane proteins can induce deleterious effects on overlying retinal pigment epithelial cells in studies relevant to age-related macular degeneration. The present work was undertaken in order to confirm 3-nitro-tyrosine (3-NT) as a product of the reaction and to identify the site specificity of nitration in collagen IV, a major component of basement membranes. Human collagen type IV was modified via incubation with 200 mM NaNO₂ (pH = 7.38) for one week at 37°C. The modified protein was prepared in 2 different ways, including acid hydrolysis and trypsin digestion for site specificity determination. The samples were analyzed by LC/MS using a C₁₂ RP column. Site specificity was determined from tandem MS/MS data utilizing TurboSEQUEST software and the Swiss-Prot sequence database. 3-NT was detected in protein digests and acid hydrolysates of nitrite modified collagen IV. Positive identification with standard 3-NT was confirmed by identical R_t, λ_{max} = 279 nm and 355 nm, and m/z = 227. Analyses of tryptic digests identified four sites of tyrosine ni-

tration, α1(IV)Y348, α1(IV)Y534, α2(IV)Y327, and α2(IV)-Y1081. These sites are located in the triple-helical region of the protein and provide clues regarding potential sites for nitrite modification in collagen type IV.

Keywords: 3-nitrotyrosine; Bruch membrane; collagen type IV; inflammation; nitrites

Introduction

Reactions involving nitrite ion are becoming increasingly recognized as a means through which oxides of nitrogen (including nitric oxide and nitrogen dioxide) exert negative effects on human tissues. Nitrite can act as a reactive substrate for an array of chemical reactions and include interactions with H⁺ (as nitrous acid), H₂O₂ (through heme peroxidase or free metals), and UV light. At least three different reaction pathways are known to occur and have biological significance. First, nitrite can mediate nitrosation reactions through the formation of nitrosonium ion (NO⁺) and/or dinitrogen trioxide (N₂O₃) in acidic electrophilic additions. Secondly, oxo-metal complex formation by heme peroxidase/H₂O₂ or free Fe/H₂O₂ in Fenton-type reactions, can cause nitration through single electron transfer to nitrite forming nitrogen dioxide (NO₂[•]) (Thomas *et al.*, 2002; Radi, 2004). Finally, hydroxyl radical (OH[•]) and nitrogen dioxide (NO₂[•]) can be produced through the photochemical decomposition of nitrite and is pH and oxygen dependent (Vione *et al.*, 2002). Thus, nitrite can be involved in both nitrosation as well as nitration reactions. In addition, nitrite has recently been proposed to function as a hypoxic nitric oxide storage and transport molecule via interactions with heme proteins such as hemoglobin and myoglobin (Dejam *et al.*, 2004). Lastly, non-enzymatic age-related modifications to the extracellular matrix proteins, such as collagen and elastin, by nitrite at physiological pH have also been studied. Nitrite was found to disrupt elastin structures, cause protein cross-linking and yellowing (Paik *et al.*, 1997; 2001). Based on UV-Vis absorption, the authors also reported that nitrite can modify tyrosine residues in type I collagen and form 3-NT (Paik *et al.*, 2001).

A significant body of current literature has accrued concerning the detection of 3-nitro-tyrosine (3-NT) in human diseases. Phenolic compounds such as tyro-

sine provide a prime reaction site for nitration reactions. Although there is significant debate as to the nitration species of greatest importance (i.e. peroxynitrite versus nitrite/H₂O₂), it is well accepted that 3-NT does form in human tissues (Dedon and Tannenbaum, 2004). Human diseases in which 3-NT has been detected are numerous and include atherosclerosis and various neurodegenerative disorders including Alzheimer's dementia (Beckman and Koppenol, 1996; Castegna *et al.*, 2003). Surprisingly, there have been no studies reported that have examined the presence of 3-NT in Bruch's membrane, although tyrosine nitration has been shown to occur in photoreceptor cells (Miyagi *et al.*, 2002). Furthermore, site specificity for tyrosine nitration has been reported for reactions with several different substrates including human serum albumin (Jiao *et al.*, 2001; Nikov *et al.*, 2003; Willard *et al.*, 2003), human angiotensin II (Petersson *et al.*, 2001), and complex I subunits of mitochondrial membranes (Murray *et al.*, 2003). Of note, site specificity data for tyrosine nitration in collagen has not previously been reported.

Type IV collagen is one of several matrix molecules present in basement membranes. This is the only collagen that has been definitively identified in the basement membrane (Linsenmayer, 1991). The interactions at the C-terminal domain of two monomers result in the formation of a dimer. Tetramers can also be formed by interactions at the N-terminal region, resulting in a highly disulfide-cross-linked 7S domain. These two types of interactions combine to produce a network-like structure (Timpl *et al.*, 1981). As such, modifications to type IV collagen may alter these three-dimensional organizations. The staining for type IV collagen in Bruch's membrane occurs in a bilaminar fashion, below the RPE cells and around the choriocapillaris (Campochiaro *et al.*, 1986). Because of the close spatial relationship, modifications to type IV collagen could impart deleterious effects on RPE cell behavior. Recent studies indicate that nitrite-modification of basement membrane-like ECM proteins (including collagen type IV) can impart deleterious effects on adjacent epithelial cell function and viability (Wang *et al.*, 2005). The basis for this cell-matrix interaction could involve cell signaling pathways through 3-NT formation. Other investigators have shown that protein nitration of fibronectin, a glycoprotein found in the extracellular matrix, can modulate fibroblast migration (Sato *et al.*, 2001). Baldus *et al.* have also shown that myeloperoxidase, the enzyme necessary for oxidation of nitrite to NO₂[•], can accumulate in the subendothelial matrix of vascular tissues to catalyze fibronectin nitration (Baldus *et al.*, 2001).

The following experiments were carried out in

order to study nitrite-mediated chemical modifications to one of the important components in basement membranes, collagen IV. The formation of 3-NT by reaction of nitrite with purified collagen type IV was confirmed and specific tyrosine residues that may be modified were identified. Several sites of tyrosine nitration were identified in the α 1(I) and α 2(I) strands of type IV collagen. All sites were located in the helical region of the collagen primary sequence.

Materials and Methods

Reagents

Sodium nitrite, acetic acid, β -mercaptoethanol and Type IV collagen (from human placenta) were purchased from Sigma Chemical Inc. (St. Louis, MO). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). All organic solvents were HPLC grade and bought from Fisher Scientific Co. (Fair Lawn, NJ).

Preparation of nitrite-modified collagen IV

A suspension of 1 mg/ml type IV collagen in 200 mM NaNO₂ or 200 mM NaCl solution (used as a control) was prepared. Both NaCl and NaNO₂ were dissolved in 10 mM pH 7.4 phosphate buffer. Samples were incubated in the dark for one week at 37°C. Excessive salts were removed by dialysis against 10 mM PBS until the solution outside the dialysis tubes was Griess assay (Bell *et al.*, 1963) negative for nitrite modification. Proteins in the dialysis tubes were collected and dried under argon and ready for further analysis.

Enzyme digestion

The modified and nonmodified type IV collagen was digested by trypsin with an enzyme-to-substrate ratio of 1:20 in pH7.4 PBS for 24 h at 37°C. The digests were then immediately subjected to LC-MS analysis.

Acid hydrolysis

Nonmodified and modified collagen IV was hydrolyzed in 6 M HCl at 110°C for 24 h using homemade glass tubes with Teflon-lined screw caps. Before hydrolysis, deoxygenation of the samples was achieved by three freeze-pump-thaw cycles. After hydrolysis, excess acid was removed using argon and samples were dissolved in 100 μ l H₂O (containing 0.05% acetic acid) and analyzed by LC-MS.

High-performance liquid chromatography-electrospray mass spectrometry (LC-ESI/MS)

HPLC separation was performed using a Synergi Max-RP C12 column (150×4.6 mm). For the enzyme digested proteins, the LC-mobile phase was CH₃CN (ACN) balanced with H₂O (both containing 0.05% acetic acid) with the following gradients: 0-2% ACN for 15 min, 2-25% ACN for 45 min, 25-95% ACN for 50 min and a flow rate 0.2 ml/min. For acid hydrolysates, the LC mobile phase was ACN balanced with H₂O (both containing 0.05% acetic acid) with the following gradients: 1-10% ACN for 50 min, 10-60% ACN for 30 min, 60-100% ACN for 20 min and a flow rate 0.2 ml/min.

The conditions for mass spectrometry (Thermo Finnigan LCQ Advantage and Surveyor LC system, San Jose, CA) were: positive polarity, capillary temperature of 200°C, source voltage of 4.5 kV,

capillary voltage of 43 V, and tube lens offset of 50 V, M/Z range: 200-1,000 (acid hydrolysis) or 300-2,000 (enzyme digest), normalized collision energy of 30%. For enzyme digests, a data-dependent method was used which contains one full MS scan followed by four MS² scans for the highest four peaks in each full scan. For acid hydrolysates, the method was set to investigate whether 3-nitro-tyrosine (m/z of [MH]⁺ is 227.1) is present in the sample or not. The method contains one zoom scan (m/z 222.1-232.1), one MS² scan with a parent mass of 227.1 and a selective reaction monitoring (SRM) scan with a parent mass of 227.1 and a fragment mass of 181.1. This fragment (m/z 181.1) corresponds to the loss of a nitro group.

The possible nitration sites were identified using SEQUEST and Swiss-Protein database. Modified sites were identified based on a mass addition of 45.

Figure 1. The Zoom Scan chromatogram of 3-NT and the acid hydrolysates of collagen IV (m/z 224.5-229.5).

Differential modifications were also considered for hydroxylation of proline and lysine. The peptides were assigned as nitrated peptides according to the following requirements:

- 1) Xcorr filter: 70% common setting: $X_{\text{corr}} > 1.3$ for +1 charged ions, $X_{\text{corr}} > 1.5$ for +2 charged ions and $X_{\text{corr}} > 2.6$ for +3 charged ions.
- 2) Amass filters (Sun *et al.*, 2004): MatchPct > 60% with RACutoff > 20%.
- 3) PDA output: the absorption spectra at the corresponding retention time is similar to the absorption spectra of 3-NT.

Levels of nitration were estimated by comparing the peak areas in zoom scan or SRM scan chromatograms of acid hydrolysates of modified collagen IV and standard 3-NT. Zoom scan and SRM scan chromatograms were first subjected to seven-point Gaussian smoothing using the Qual Browser Program of the Finnigan Xcalibur 1.3 Software and then integrated after manually choosing peaks that correspond to 3-NT. Standard 3-NT solution with a series of concentrations was injected into LC-MS. The calibration curve was obtained by plotting the peak area versus the mass of 3-NT. The amount of 3-NT in modified collagen was calculated using this calibration curve and the 3-NT peak area in chromatograms of acid hydrolysates.

Results

Nitrite modified tyrosine residues in collagen IV

In order to study the modifications to collagen IV by nitrite, collagen IV was incubated with nitrite for one week at 37°C at physiological pH (7.4). Following confirmation of complete nitrite removal using a colorimetric nitrite assay (Bell *et al.*, 1963), modified proteins were hydrolyzed by 6 M HCl. Acid hydrolysates were analyzed by LC-MS. The results indicate that tyrosine nitration by reaction with nitrite in collagen IV can form 3-nitrotyrosine (3-NT). Acid hydrolysis was used because 3-NT is stable under acid hydrolysis (Crowley *et al.*, 1998). Figure 1 gives the chromatogram of the zoom scans of different samples. The m/z of the quasimolecular ion ($[MH]^+$) of 3-NT is 227.1. The zoom scan monitored the presence of any compound with m/z 224.5–229.5. From the analysis of standard 3-NT, it is known that the retention time of 3-NT at this experimental condition is around 47 min. 3-NT in nonmodified collagen IV was not detectable under our experimental condition (Figure 1). Although there were several peaks with m/z around 227.1, the retention times of these peaks were far away from 47 min. A significant amount of 3-NT was present in the acid hydrolysate of nitrite-modified collagen IV (Figure 1).

Figure 2. The Selected Reaction Monitoring (SRM) chromatogram of 3-NT and acid hydrolysate of nitrite-modified collagen IV (SRM 227.1 $\rightarrow$ 181.1).

Figure 3. The calibration curves for quantification of 3-NT.

Selected reaction monitoring (SRM) scans were also performed in order to further verify that the peak at retention time 47 min was in fact, 3-NT. In the SRM mode, the detector was programmed to detect the presence of compounds with a parent ion of 227.1 and one fragment ion of 181.1. 3-NT has a major fragment with m/z 181.1 which corresponds to the loss of a $-NO_2$ group. The results of the SRM scan are shown in Figure 2. There is a peak in the SRM scan which indicates the presence of 3-NT in the acid hydrolysate of nitrite-modified collagen IV. This result confirms that nitrite modification of collagen IV results in the formation of 3-NT.

In order to quantitate the amount of tyrosine nitration by reaction with nitrite, different concen-

Figure 4. The tandem mass spectra of the modified peptides. The tandem mass spectrum is labeled with the predicted b and y ions. B and y ions consistent with nitration are shown with an asterisk. (A) The identified peptide for this MS/MS spectrum has the following sequence: AGLY#GEIGATGDFGDIGDTINLPGR. The modified tyrosine is Y1081 in alpha 2 chain of collagen IV. The calculated $[M + 2H]^{2+} = 1240.85$, the experimental $[M + 2H]^{2+} = 1240.93$. Xcorr: 6.58. (B) The identified peptide for this MS/MS spectrum has the following sequence: DGVAGVP@GPQGTP@GLIGQPGAK@GEP@GEFY#FDLR. The modified tyrosine is Y534 in alpha 1 chain of collagen IV. The calculated $[M + 2H]^{2+} = 1696.81$, the experimental $[M + 2H]^{2+} = 1696.56$. Xcorr: 2.83. @ shows the hydroxylation. (C) The identified peptide for this MS/MS spectrum has the following sequence: GY#P@GTP@GPR. The modified tyrosine is Y348 in alpha 1 chain of collagen IV. The calculated $[M + 2H]^{2+} = 489.73$, the experimental $[M + 2H]^{2+} = 490.23$. Xcorr: 1.45. @ shows the hydroxylation. (D) The identified peptide for this MS/MS spectrum has the following sequence: GLDGY#QGPDGPR. The modified tyrosine is Y327 in alpha 2 chain of collagen IV. The calculated $[M + 2H]^{2+} = 638.79$, the experimental $[M + 2H]^{2+} = 639.48$. Xcorr: 3.04.

trations of 3-NT solution were injected into the LC-MS. Following analysis, calibration curves were obtained from area calculations of both zoom scans and SRM scans independently. The regression equations for the calibration curves were $y = 276082861.91x + 17878361.76$ for SRM scans, and $y = 625280387.64x + 23726717.24$ for zoom scans. Utilizing these equations, the amount of 3-NT in the acid hydrolysates of nitrite-modified collagen IV were calculated. Using zoom scan data, the amount of 3-NT in modified proteins was calculated to be 9.35 $\mu\text{g}/\text{mg}$ proteins. Using SRM scan data, the amount of 3-NT in modified proteins was calculated to be 10.15 $\mu\text{g}/\text{mg}$ proteins. Thus, both methods yield comparable results. Based on the molecular weight of collagen IV and the number of tyrosine residues present in the protein, the relative amount of modified tyrosine residues was estimated to be 40%.

Identification of the sites of modified tyrosine residues

Trypsin digests of nitrite-modified collagen IV were analyzed by LC-MS. Following each full scan, four tandem mass spectra were obtained for the four highest peaks in each full scan. The MS/MS data were then extracted from the raw file and a SEQUEST algorithm was used to identify the modified peptides. A cross-correlation function (Xcorr) was then provided by SEQUEST to discriminate positive identifications from negative identifications. However, protein identification by the SEQUEST method does not take into account unusual fragmentation events that can occur as a result of the presence of specific residues. For example, the presence of proline residues in a peptide has a significant effect upon the tandem mass spectra. Protonation and cleavage of the proline amide bond by collision induced dissociation (CID) to produce a y type ion is a highly favored process and often dominates the tandem mass spectra (Hunt *et al.*, 1986). As a result, it is difficult to sequence proline-rich proteins by CID mass spectrometry. Because prolines are abundant in collagen, identification of these collagen peptides can be a difficult process. Therefore, when the modified peptides were searched, we decreased the requirement for Xcorr to 70% of the common setting. In addition, SEQUEST parameters were combined with manual validation and other parameters such as Amax. Based on these methods, four modified peptides were identified. Figure 4 (A-D) give the tandem mass spectra for these identified peptides. The identified sites of tyrosine modification were Y348, Y534 in the $\alpha 1$ chain and Y327, Y1081 in the $\alpha 2$ chain of collagen IV. All of these residues are in the triple

Figure 5. The PDA output at the retention time of the modified peptide D, standard 3-NT and the nonmodified peptides at the same retention time.

helical region of collagen IV. In addition, the absorption spectra for each of the above peptides were similar to that of standard 3-NT, consistent with the presence of 3-NT in each peptide. Figure 5 is an example of the absorption spectrum of one of these peptides.

The most common molecular form of type IV collagen is believed to be a heteropolymeric molecule of the structure $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$. However, it has also been suggested that the homopolymeric forms $[\alpha 1(\text{IV})]_3$ and $[\alpha 2(\text{IV})]_3$ occur as well. In addition, at least 4 additional type IV collagen molecules are now known to exist (Linsenmayer, 1991). Using Xcorr values chosen to satisfy the common setting, when the database contains $\alpha 1$ or $\alpha 2$ chains only, more than 20 positively matched peptides for each chain were identified. However, when the database contains $\alpha 3$, $\alpha 4$, $\alpha 5$, or $\alpha 6$ chains, no peptide matches from the tandem mass spectra of LC-MS analyses were found. This result indicated that the collagen IV used in these experiments was mainly a heteropolymeric molecule of the structure $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$.

In addition to the four mentioned sites, other tyrosine residues could be modified as well. As mentioned previously, the high abundance of proline in collagen can make the identification of peptides by SEQUEST difficult. When these SEQUEST results were carefully examined, several peptides with low Xcorr number (around 1.0), contained matches for all of the high-abundance fragment ions of the tandem mass spectra with some continuity of the b,y ions. Thus, these peptides could have been modified as well. These peptides include Y1107, Y1352 and Y675 in the $\alpha 1$ chain, and Y351 in the $\alpha 2$ chain.

Discussion

Collagen IV is an important ECM protein in Bruch's membrane. Post-translational modifications to collagen IV can affect the function of this protein. Our previous experiments indicate that reaction with nitrite increases collagen cross-linking and yellowing (Paik *et al.*, 2001; 2006). In this current study, site specificity of tyrosine nitration in collagen IV has been determined. About 40% of the available tyrosine residues underwent nitration. Since it is known that there are 18 tyrosine residues in the $\alpha 1$ chain and 13 tyrosine residues in the $\alpha 2$ chain, at least 7 residues in the $\alpha 1$ chain and 5 residues in the $\alpha 2$ chain were modified. There are two reasons why additional, as yet unidentified, sites of tyrosine nitration may exist. The first reason has been described previously and deals with the difficulty of identifying proline-rich peptides by SEQUEST. Second, modification to collagen IV by nitrite results in the formation of covalent cross-links within the protein. Once the protein is cross-linked, the sequence of the protein will be changed. Because SEQUEST utilizes the original protein sequence in order to identify the modified peptides, areas involved in the formation of cross-links that contain sites of tyrosine nitration will not be accurately identified.

Nitration of protein tyrosine residues to form 3-NT is considered a hallmark of tissue injury caused by inflammation. 3-NT has been identified in many diverse pathological conditions such as atherosclerosis, pulmonary and heart disease, viral infections and neurological disorders (Ischiropoulos, 1998). Recent studies have established that in addition to serving as a "marker" of reactive nitrogen species (RNS) formation, nitration of key tyrosine residues can alter the function of proteins. For example, modification of tyrosine residues can affect the phosphorylation and dephosphorylation of tyrosine, an important mechanism of cell regulation (Di Stasi *et al.*, 1999; MacMillan-Crow *et al.*, 2000). In addition, nitration of tyrosine in the rat skeletal-muscle sarcoplasmic reticulum Ca^{2+} ATP-ase (SERCA2a) enzyme may affect its catalytic activity (Viner *et al.*, 1999). Lastly, incorporation of free 3-NT into proteins through translation has been reported to alter microtubule function (Eiserich *et al.*, 1999).

Despite mounting evidence for the physiologic and pathophysiologic relevance of protein nitration, the mechanisms underlying this modification *in vivo* are still poorly understood. It was initially believed that 3-NT was formed solely by the reaction of tyrosine with peroxynitrite, a potent oxidant resulting from the diffusion limited reaction of nitric oxide (NO) with superoxide anion (O_2^-) (Koppenol *et al.*, 1992). Based on this assumption, 3-NT has been used as a

specific biomarker for peroxynitrite production (Moriel and Abdalla, 1997; Hoeldtke *et al.*, 2003). However, investigators have questioned the plausibility of the peroxynitrite mechanism (Fukuto and Ignarro, 1997). For example, tyrosine nitration has been shown to be very inefficient when NO and O_2^- are generated at equal rates at physiological pH (Pfeiffer and Mayer, 1998). Several other peroxynitrite-independent mechanisms for 3-NT formation have been identified (Eiserich *et al.*, 1996; Van der Vliet *et al.*, 1997) and several excellent reviews have been recently published (Bian *et al.*, 2003; Whiteman *et al.*, 2003; Dedon and Tannenbaum, 2004). The myeloperoxidase (MPO) catalyzed nitration has received considerable attention (Van der Vliet *et al.*, 1997; Pfeiffer *et al.*, 2001) and proceeds through a Fenton type mechanism involving one electron oxidation of nitrite to form nitrogen dioxide which then modifies the substrate. MPO is a heme protein abundantly expressed in polymorphonuclear neutrophils and monocytes during inflammation and, thus, NO_2^- and MPO can be produced simultaneously during an inflammatory process.

In contrast to these previously noted studies, we have focused primarily on the endpoint changes that can occur to long-lived proteins as a result of reaction with nitrite and the effect that this can have on cell and tissue function. Because these modifications are accumulated over decades in the living organism, it is necessary to use nitrite concentrations that are higher than those found under physiological conditions. The maximum serum concentrations that have been reported in the literature are on the order of micromolar concentrations (Radi, 2004). In this regard, we have developed a model system patterned on the well known model system of Non-Enzymatic Glycation (NEG) in which long-term incubations utilizing greater than physiologic concentrations of reactants are used to study effects that could have potentially taken decades to develop.

NEG is a well established aging model system relevant to diabetic-related complications and aging. It is well known that the level of reactants used to study the effects of glycation on connective tissue proteins (i.e. 100-250 mM) are well above those encountered *in vivo* (Kohn *et al.*, 1984; Fu *et al.*, 1994; Winlove *et al.*, 1996). The rationale behind the use of supraphysiologic reactant concentrations is to identify the end-products of the reaction and to simulate functional, biomechanical, and biochemical changes that could take decades to develop *in vivo*. The work which identified pentosidine as a cross-link biomarker of collagen glycation utilized concentrations of 100 mM pentoses for an incubation period of 6 days (Sell and Monnier, 1989). Subsequently,

several products of these *in vitro* incubations have been detected *in vivo*, including advanced glycation end-products (AGEs) such as pentosidine (Sell and Monnier, 1989; Sims *et al.*, 1996) and the adduct carboxymethyl lysine (Dyer *et al.*, 1993). Thus, the presence of these advanced glycation end-products *in vivo* have established the biological relevance of NEG and have validated this experimental method as a means for studying long-term chemical effects to connective tissue proteins such as collagens.

Reactions involving nitrous acid, the conjugate acid of nitrite, have been well studied in the past. Electrophilic addition reactions with various substrates including proteins and amino acids were described during the 1970's. During that time, reactions with secondary amines received particular attention since their primary products (i.e. N-nitroso compounds) were found to be highly carcinogenic and mutagenic (Knowles *et al.*, 1974; Ohara *et al.*, 1988). Modifications to proteins by nitrite at neutral pH, in contrast, were not widely studied. This may have been in part due to the fact that the rate of reaction at neutral pH is very slow. Thus, neutral reactions may not have been thought to be important at that time. However, it is now known that during chronic inflammation, tissues may be exposed to elevated nitrite levels for prolonged periods. Ongoing elevated nitrite exposure at neutral pH could occur and thus, the reactions at neutral pH may be relevant to *in vivo* processes involving long-lived extracellular proteins. It is not clear at this time whether such reactions that are carried out at neutral pH are in fact occurring through nitrous acid formation. Even at neutral pH, solutions containing high nitrite concentrations will contain small amounts of nitrous acid which could be consumed during reaction incubations. Further study will be required in order to more fully delineate the mechanism of reaction in our model system and *in vivo*.

Importantly, the results described have indicated that a by-product of inflammation, nitrite, can damage the structure of human basement membranes. Chronic inflammation, a state characterized by increased oxidative and nitrosative stress, has been strongly implicated in the development of AMD. Hageman *et al.* have clearly identified the BM region as a site of active inflammation (Anderson *et al.*, 2002). Furthermore, recent exciting information concerning the genetics of AMD indicates that alterations in the complement pathway of inflammation plays a major role in the disease (Daiger, 2005). Four independent studies, published this year, implicate complement factor H, an inhibitor of uncontrolled complement activation via the alternative complement pathway (Edwards *et al.*, 2005; Hageman *et al.*, 2005; Haines *et al.*, 2005; Klein *et al.*,

2005). Upregulation of the alternative pathway of complement activation will promote the formation of reactive nitrogen species (RNS), including by-products of nitric oxide formation, such as nitrite. This would provide a prime environment for the formation of both nitrating and nitrosating species capable of connective tissue protein damage through non-enzymatic collagen cross-linking and elastin fragmentation.

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