Nitric oxide inhibits the synthesis of type-II collagen without altering Col2A1 mRNA abundance: prolyl hydroxylase as a possible target

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The addition of human recombinant interleukin-1β (IL-1β) to cultures of lapine articular chondrocytes provoked the synthesis of large amounts of NO and reduced the production of type-II collagen. N^G-Monomethyl-L-arginine (L-NMA), an inhibitor of NO synthase, strongly suppressed the production of NO and partially relieved the inhibition of collagen synthesis in response to IL-1β. The NO donor *S*-nitrosoacetylpenicillamine (SNAP), on the other hand, inhibited collagen production. IL-1 lowered the abundance of Col2A1 mRNA in an NO-independent manner. Collectively, these data indicate that IL-1 suppresses collagen synthesis at two levels: a pretranslational level which is NO-independent, and a translational or post-translational level which is NO-mediated. These effects are presumably specific as L-NMA and SNAP had no effect on total protein synthesis or on the distribution of newly synthesized proteins between the cellular and extracellular compartments. Prolyl hydroxylase is an important enzyme in the post-translational processing of collagen, and its regulation and cofactor requirements suggest possible sensitivity to NO. Extracts of cells treated with IL-1 or SNAP had lower prolyl hydroxylase activity, and L-NMA was partially able to reverse the effects of IL-1. These data suggest that prolyl hydroxylase might indeed be a target for NO. Because under-hydroxylated collagen monomers fail to anneal into stable triple helices, they are degraded intracellularly. Inhibition of prolyl hydroxylase by NO might thus account for the suppressive effect of this radical on collagen synthesis.

INTRODUCTION

NO is a radical synthesized in exceedingly high amounts by activated articular chondrocytes of all animal species so far tested, including man [1–4]. NO production requires activation of chondrocytes by agents such as interleukin 1 (IL-1), tumour necrosis factor α, leukaemia inhibitory factor and endotoxin. These stimuli provoke the synthesis of an inducible isoform of NO synthase, which generates large amounts of NO for a sustained period of time [1,4–6]. Data from studies conducted with experimental animals implicate NO in the pathophysiology of the arthritic joint [7–10]. Moreover, *ex vivo* analysis of human arthritic cartilage indicates that NO synthase is expressed in the chondrocytes of diseased, but not normal, tissue [11–13].

In seeking a role for this radical in cartilage metabolism, it has been noted that endogenously generated NO suppresses the biosynthesis of aggrecan, a major macromolecular component of the cartilaginous matrix, in cartilage derived from rabbits [14], humans [15,16] and rats [17]. Moreover cartilage derived from the inducible NO synthase knockout mouse shows a strongly blunted response to IL-1 in terms of suppressed proteoglycan synthesis (J. Mudgett, personal communication). These responses are likely to be of pathophysiological importance, as proteoglycan synthesis is strongly suppressed in the articular cartilages of animals with experimental models of rheumatoid arthritis and is reduced in human cartilage treated with IL-1 *in vitro* [18,19]. Because most samples of human articular cartilage mount a poor catabolic response to IL-1 [19–21], suppression of matrix synthesis may be a particularly critical pathophysiological event in human arthritis.

In addition to proteoglycan, the integrity of the cartilaginous matrix depends crucially on the presence of type-II collagen. This forms a fibrous meshwork within which the large aggregating proteoglycans are restrained, and it also provides tensile strength to the tissue [22]. Synthesis of this important macromolecular matrix constituent by chondrocytes is also inhibited by IL-1 [23,24], but the possible role of NO in this response has not been previously studied. The present investigation was undertaken to determine the possible effect of NO on the synthesis of collagen by chondrocytes and to assess the degree to which NO might account for the suppressive effect of IL-1 in this regard.

Here we report that NO inhibits type-II collagen synthesis by rabbit articular chondrocytes at a translational or post-translational level. Evidence consistent with the hypothesis that prolyl hydroxylase is an important target for NO is also presented.

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated suppliers: New Zealand rabbits (2.3–2.7 kg) (Green Meadows Rabbitry, Murraysville, PA, U.S.A.); tissue culture media, sera, antibiotics, etc. (Gibco, Grand Island, NY, U.S.A.); chromatographically purified collagenase, form III (Advanced Biofactures Corp., Lynbrook, NY, U.S.A.); crude clostridial collagenase

Abbreviations used: IL-1, interleukin 1; rhIL-1β, recombinant human interleukin 1β; L-NMA, N^G-monomethyl-L-arginine; SNAP, *S*-nitrosoacetylpenicillamine.

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(Worthington Biochemical Corporation, Freehold, NJ, U.S.A.); L-[2,3-^{3}H]proline (55 Ci/mmol), [^{35}S]methionine (1175 Ci/mmol) and Enhance (DuPont, Boston, MA, U.S.A.); multiprime DNA labelling system (Amersham, Arlington Heights, IL, U.S.A.). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). N^G-Monomethyl-L-arginine (L-NMA) was synthesized by Dr. Paul Dowd and Dr. Wei-Zhang of the Department of Chemistry, University of Pittsburgh, PA, U.S.A., and (Pro-Pro-Gly)$_{10}$ was synthesized by the Peptide Facility of the Pittsburgh Cancer Institute. A 452 bp PCR fragment derived from a human cDNA clone (pCAR1) used to probe Col2A1 mRNA was a gift from Dr. C. Baldwin, Boston University School of Medicine, Boston, MA, U.S.A.; *S*-nitrosoacetylpenicillamine (SNAP) and recombinant human interleukin 1β (rhIL-1β) were gifts from Dr. David Geller, Department of Surgery, University of Pittsburgh School of Medicine, and Elizabeth Arner of DuPont Merck, Wilmington, DE, U.S.A. respectively.

Chondrocyte culture

Monolayer cultures of lapine articular chondrocytes were prepared in 24-well or 6-well plates, as necessary, by the method of Green [25]. Cells were grown in Ham's F12 medium supplemented with 10 % fetal bovine serum and antiboitics, and used without subculture. As confirmed in the present study, cells in such cultures retain their differentiated type-II collagen phenotype.

At confluence, ascorbic acid (50 μg/ml) was added and 24 h later the growth medium was discarded. Neuman–Tytell medium, which contains 0.2 % lactalbumin hydrolysate in place of serum, was then added. This medium was supplemented with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml) for studies of collagen synthesis. At this time were added rhIL-1β (20 units/ml) in the presence or absence of L-NMA (0.5 mM), SNAP (100 μM) or other reagents, as indicated in the text. We have previously shown that this concentration of SNAP drives NO-dependent processes in chondrocytes without causing toxicity [14,16].

Collagen synthesis

Collagenase method

In the first method, the incorporation of [^{3}H]proline into collagenase-sensitive proteins was measured by a modification [26] of the method of Peterkofsky and Diegelmann [27]. Briefly, ascorbic acid (50 μg/ml) was added to confluent cultures of chondrocytes for 24 h before removal of growth medium and its replacement with Neuman–Tytell medium containing [^{3}H]proline (5 μCi/ml), ascorbic acid (50 μg/ml), β-aminopropionitrile (50 μg/ml) and IL-1 or L-NMA with or without SNAP as appropriate. After a further 24 h incubation, medium was removed, the cell sheet lysed into distilled water by freezing and thawing, and then combined with the medium. After extensive dialysis of the combined medium and cell lysates against distilled water, samples were incubated for 150 min at 37 °C in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mg/ml NaN_3, 0.05 % (v/v) Triton X-100 and 3 mM *N*-ethylmalemide in the presence or absence of highly purified bacterial collagenase (2.5 units/ml; Advanced Biofactures Corp.) or a mixture of crude trypsin (100 μg/ml) and crude bacterial collagenase (100 μg/ml; Worthington Biochemical Co.). Undigested proteins were precipitated with cold 10 % (w/v) trichloroacetic acid in the presence of BSA (1 mg/ml). After centrifugation [20000 ***g***; 1 h)], radioactivity in the supernatant was measured by liquid scintillation.

Release of radioactivity by the highly purified collagenase was taken as a measure of collagen biosynthesis; release of radioactivity by the mixture of crude collagenase and crude trypsin was taken as a measure of total protein synthesis. Subtraction of these two values provided a measure of the biosynthesis of non-collagenous proteins, as described by Peterkofsky and Diegelmann [27].

Electrophoretic method

Samples were prepared as described above, except that the combined lysates and conditioned media were digested with pepsin (100 μg/ml) dissolved in 0.5 M acetic acid at 4 °C for 4 h. The pepsin-resistant proteins were dialysed against 0.05 M NH_4HCO_3 and lyophilized. The pellets were redissolved in an equal volume of electrophoresis buffer, and aliquots (60 μl) subjected to SDS/PAGE using 6 % gels. Type-II collagen was purified from bovine articular cartilage, and used as a standard. After electrophoresis, gels were dried and radioactive proteins were detected by autoradiography.

Specific radioactivity of intracellular proline pool

After adding radiolabelled proline to the cultures as described above, the cell layers were washed three times with PBS at 4 °C and then harvested by scraping into 1 ml of 10 % (w/v) trichloroacetic acid. These lysates were stored overnight at 4 °C and then centrifuged (12000 ***g***; 10 min). The supernatants, containing the intracellular pools of free proline, were filtered (0.45 μm cut-off), vacuum dried and resolubilized in 200 μl of 'Buffer S' (Beckman Instruments).

Aliquots (50 μl) of each sample were fractionated on a Beckman System 6300 amino acid analyser. Fractions (0.5 ml) were collected every minute, and those containing amino acids were identified by the ninhydrin reaction. The concentration of proline in each aliquot was calculated from the area under the curve of the proline peak, and its radioactivity measured by scintillation counting. Specific radioactivity was expressed as c.p.m./nmol.

Total protein synthesis and secretion

As an additional measure of total protein synthesis, [^{35}S]methionine (15 μCi/ml) was added to the cultures 20 h after the addition of IL-1 with or without L-NMA or SNAP. After 4 h further incubation, media were separated from the cell sheet, which was washed with PBS and lysed into distilled water. The media and cell lysates were adjusted to 10 % trichloroacetic acid and kept on ice for 1 h to precipitate proteins. Precipitates were collected by centrifuging, washed with 5 % trichloroacetic acid and 100 % ethanol, filtered and their radioactivities determined by liquid scintillation.

For SDS/PAGE analysis of cell-associated and secreted proteins, conditioned media were dialysed, dried and redissolved in electrophoresis buffer. The cell sheet was lysed directly into electrophoresis buffer. Aliquots (60 μl) were separated on 6 % polyacrylamide gels, and radioactive bands determined by autoradiography.

Northern-blot analysis

RNA was extracted using the acid/phenol/guanidinium isothiocyanate method of Chomczynski and Sacchi [28]. RNA concentrations were determined by spectrophotometrical analysis at 260 nm, and 8 μg of RNA per lane was fractionated by electrophoresis through 1 % agarose/formaldehyde gels. RNA was transferred by capillary force to nylon membranes for 16–20 h

and UV-cross-linked to the filter. DNA probes were labelled with [α^{32}P]dATP by the random-primer method to yield specific radioactivities of (1.5–2) $\times 10^9$ d.p.m./mg.

After 4–6 h of prehybridization, membranes were hybridized overnight at 65 °C using a phosphate buffer system with SDS [29]. Membranes were washed twice at 60 °C before exposure to X-ray film at −80 °C. Radioactive bands were detected autoradiographically and the relative intensities of the bands were determined by densitometric scanning using a Bio-Rad (model FS-670) densitometer.

Prolyl hydroxylase assay

The method of Kivirikko and Myllylä [30] was used to measure prolyl hydroxylase. Prolyl hydroxylase was extracted from chondrocytes by homogenizing them in enzyme buffer containing 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 0.1 % Triton X-100 and 0.01 M Tris/HCl, pH 7.8, at 4 °C. Synthetic polypeptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ was used as substrate. Enzyme extract (1 ml) and the other reactants (50 mM Tris/HCl, pH 7.8, 0.1 mg/ml catalase, 2 mg/ml BSA, 0.05 mM $FeSO_4$, 2 mM ascorbic acid, 0.1 mM dithiothreitol, 0.5 mM 2-oxoglutaric acid) were added. Distilled water was added to adjust the final volume to 2.0 ml. After incubation at 37 °C for 1 h, the samples were hydrolysed in 6 M HCl for 16 h at 108 °C and dried *in vacuo*. The hydroxyproline content of the dried samples was measured by the method of Woessner [31].

Nitrite determination

NO production was determined by measuring the NO_2^- concentration of the conditioned medium by a spectrophotometric assay based on the Griess reaction [32]. We have previously confirmed that approx. 50 % of the NO produced by chondrocytes in culture accumulates as NO_2^- over a wide range of NO production levels [1].

RESULTS

Effect of NO on collagen synthesis

IL-1 was used to provoke NO biosynthesis. As shown in Table 1, addition of IL-1 led to enhanced NO production and a 47 % inhibition of the incorporation of [^{3}H]proline into collagenase-sensitive proteins. Addition of L-NMA inhibited NO biosynthesis and restored collagen synthesis to approx. 80 % of its control level. Addition of SNAP led to the release of large amounts of NO and inhibited collagen synthesis by 39 %. Measurement of the specific radioactivity of the intracellular pool of radiolabelled proline (Table 2) confirmed that changes in this parameter were not responsible for the observed alterations in the incorporation of [^{3}H]proline into collagen. These data thus suggest that NO inhibits the synthesis of collagen and accounts substantially, but not completely, for the suppression of collagen synthesis in response to IL-1.

To confirm that these responses were not an artifact of monolayer culture, the experiment was repeated with slices of otherwise intact lapine articular cartilage. The same result was obtained (results not shown).

SDS/PAGE analysis (Figure 1) confirmed that control chondrocytes produced type-II collagen, with no evidence of α2(I) chains or other collagen molecules that would suggest dedifferentiation. This analysis did, however, reveal the presence of faint bands that co-migrate with the α1 chains of type-V and

Table 1 Effects of IL-1, L-NMA and SNAP on nitrite accumulation and the incorporation of [^{3}H] proline into collagenase-sensitive proteins

Values given are means ± S.E.M. *Values differ from control values, $P < 0.05$; †values differ from IL-1-treated samples, $P < 0.05$ (Student's t test).

Addition to culture	[Nitrite](μM) ($n = 8$)	Collagenase-sensitive [^{3}H]Pro incorporation (% of control) ($n = 15$)
None (control)	2.5 ± 0.5	100
IL-1	19.3 ± 1.6*	53 ± 7*
IL-1 + NMA	5.8 ± 0.4†	81 ± 3†
SNAP	35.5 ± 3.8†	61 ± 6*

Table 2 Specific radioactivity of intracellular unincorporated proline under various culture conditions

Values given are the means of two independent determinations.

Addition to culture	Specific radioactivity (c.p.m./nmol)
None (control)	5745
IL-1	6217
IL-1 + NMA	6937
NMA	6017
SNAP	6760

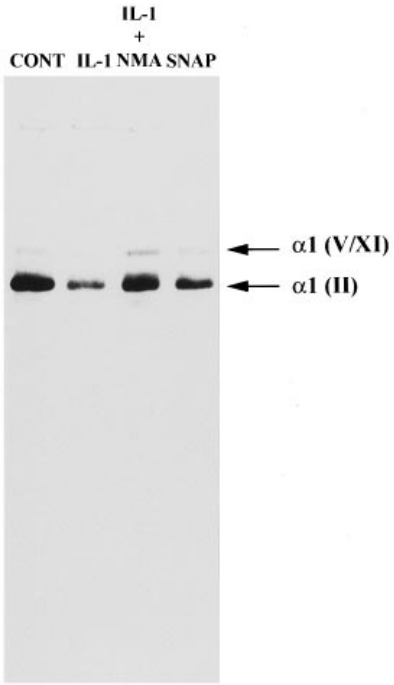

Figure 1 Autoradiogram demonstrating the effects of IL-1, L-NMA and SNAP on the incorporation of [^{3}H]proline into the α-chains of collagens synthesized by articular chondrocytes

Monolayers were labelled with [^{3}H]proline in the presence or absence of IL-1, L-NMA and SNAP. Proteins were digested with pepsin, separated by SDS/PAGE and the dried gels subjected to autoradiography as described in the Materials and methods section. CONT, control.

Table 3 Synthesis of protein by chondrocytes in the presence or absence of IL-1, L-NMA and SNAP

Values are means ± S.E.M. from six determinations. There is no statistically significant effect of IL-1, L-NMA or SNAP on the incorporation of [^{3}H]proline into total non-collagenous proteins or on the incorporation of [^{35}S]methionine into cell-associated proteins, secreted proteins or total (cells + medium) proteins.

Addition to culture	[^{3}H]Proline incorporation into non-collagenous proteins (c.p.m.)	[^{35}S]Methionine incorporation (c.p.m.)		
		Cell	Medium	Total
None	66348 ± 6000	31240 ± 1852	10833 ± 2498	42073 ± 3480
IL-1	55554 ± 3272	27299 ± 2230	12080 ± 3193	39379 ± 5069
IL-1 + L-NMA	70833 ± 4356	28018 ± 2380	11738 ± 2588	39756 ± 4942
SNAP	63788 ± 5404	31983 ± 1386	8591 ± 1168	40574 ± 1611

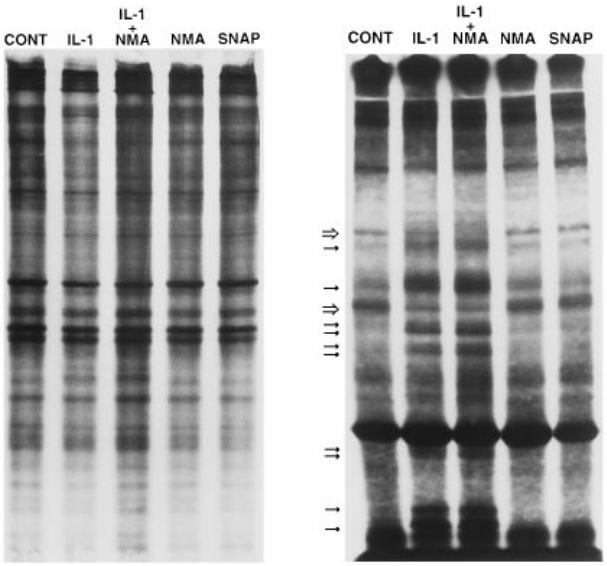

Figure 2 Autoradiograms of [^{35}S]methionine-labelled proteins from the medium and cells of cultures incubated with IL-1, L-NMA or SNAP

Left, cell lysates; right, media. Closed arrows indicate the positions of proteins the synthesis of which was increased by IL-1. Open arrows indicate proteins that were decreased by IL-1. CONT, control.

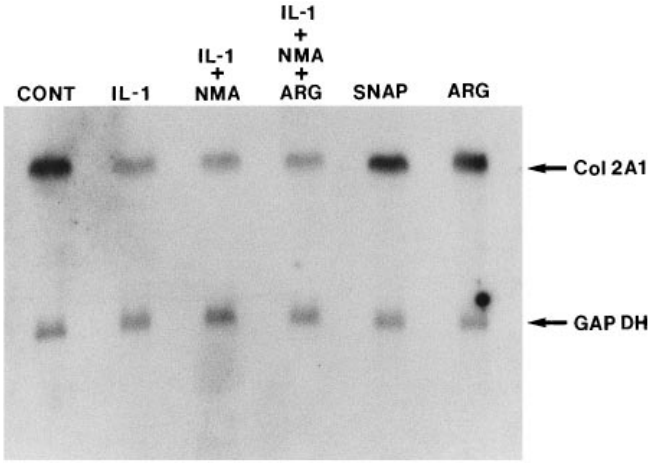

Figure 3 Northern blot demonstrating the effects of IL-1, L-NMA, SNAP and arginine on the abundance of Col2A1 mRNA in chondrocytes

Chondrocytes were incubated for 24 h with rhIL-1β (20 units/ml), L-NMA (0.5 mM), SNAP (100 μM) or L-arginine (10 mM), either singly or in the indicated combinations. RNA was then extracted and Northern-blot analysis performed as described in the text. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

type-XI collagen, which are found in articular cartilage. Addition of rhIL-1β or SNAP strongly suppressed the synthesis of type-II and type-V/XI collagen; inhibition by IL-1 was relieved by the addition of L-NMA.

Effects of NO on total protein synthesis and secretion

The incorporation of [^{3}H]proline into non-collagenous proteins and the incorporation of [^{35}S]methionine into total protein were affected to only a small degree by IL-1, L-NMA and SNAP (Table 3). Furthermore there was no skewing of the distribution of radiolabelled proteins between the medium and the cell sheet (Table 3).

No obvious differences in the SDS/PAGE pattern of [^{35}S]methionine-labelled intracellular proteins existed between control cells and those incubated with IL-1 in the absence or presence of L-NMA or SNAP (Figure 2, left). Inspection of autoradiograms prepared from the conditioned media revealed a number of proteins, the synthesis of which was increased by IL-1, and a number for which synthesis was inhibited (Figure 2, right). However, neither L-NMA nor SNAP influenced this pattern of IL-1-mediated changes. Procollagen and collagen contain very little methionine, and consequently do not appear on these autoradiograms.

Effects on mRNA abundance

As shown by the representative blot in Figure 3, IL-1 strongly reduced the abundance of Col2A1 mRNA. This was not reversed by the addition of L-NMA, and addition of SNAP alone failed to reduce mRNA abundance. This indicates that NO inhibits type-II collagen synthesis at a translational or post-translational level. Hybridization with a cDNA directed against mRNA encoded by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase confirmed that these differences in Col2A1 mRNA abundances were not due to uneven loading of the gels.

Effects of NO on prolyl hydroxylase activity

Translational inhibition by NO seemed less likely than post-translational inhibition, as the former could not easily account for the specificity noted in the present work. Of the various post-translational processing steps undergone by collagen, prolyl

Table 4 Effect of IL-1, L-NMA and SNAP on the activity of prolyl hydroxylase in extracts of chondrocytes

Results are means ± S.E.M. *Values differ from control values, $P < 0.05$; †values differ from IL-1-treated samples, $P < 0.05$ (Student's t test).

Addition to culture	Activity (μg of hydroxyproline incorporated/10^6 cells)
None (control)	2.3 ± 0.4
IL-1	0.5 ± 0.2*
IL-1 + NMA	1.2 ± 0.3†
SNAP	0.8 ± 0.4*

hydroxylase seemed a promising target for inhibition by NO. As shown in Table 4, prolyl hydroxylase activity was inhibited 70% by IL-1 and 60% by SNAP. The inhibitory effect of IL-1 was partially relieved by the addition of L-NMA.

DISCUSSION

These data clearly demonstrate that NO inhibits the synthesis of collagen by lapine articular chondrocytes. We know of only two other publications, both of them short phenomenological reports, on the effect of NO on collagen synthesis. One, which used smooth-muscle cells, noted a profound inhibition of collagen synthesis, but this seemed to be a non-specific consequence of an equally profound inhibition of total protein synthesis by NO [33]. The other, using mesangial cells, noted a 35% suppression of the incorporation of radiolabelled proline into collagenase-sensitive proteins by cells exposed to NO [34]. In this case the effect showed some specificity in that the synthesis of laminin was considerably enhanced by NO.

Our results are consistent with the findings of Trachtman et al. [34], who, using mesangial cells, noted a similar degree of inhibition of collagen synthesis by endogenously generated NO. This is a specific effect, as neither total protein synthesis nor the distribution of newly synthesized proteins between the chondrocytes and the medium is altered by NO. Lapine articular chondrocytes thus differ from lapine smooth-muscle cells [25] and human hepatocytes [35], the total protein synthesis of which is sensitive to NO.

Unlike IL-1, NO fails to reduce the abundance of Col21A mRNA. This explains why L-NMA gives only partial relief from the inhibition of collagen synthesis imposed by IL-1: blocking NO production by chondrocytes treated with IL-1 restores translational/post-translational events but fails to increase mRNA abundance. It also provides a possible mechanism whereby interferon γ inhibits collagen synthesis by chondrocytes [36] without generating NO [1]. This cytokine presumably acts by reducing mRNA abundance without affecting later events.

NO could inhibit collagen biosynthesis at the level of translational or post-translational processing. The former possibility is less likely, as it is hard to envisage how translational inhibition could be specific for collagen. Post-translational events, however, are good candidates as collagen undergoes an unusually large number of complex processing steps [37]. Moreover, when these are impaired the collagen does not anneal correctly into triple helices; under these conditions, collagen is degraded intracellularly [38].

Of the post-translational processing steps undergone by collagen, the hydroxylation of proline residues by prolyl hydroxylase would seem *a priori* most vulnerable to NO. Moreover, the activity of prolyl hydroxylase usually correlates with the rate of collagen synthesis [39,40]. Prolyl hydroxylase requires Fe^{2+} and ascorbate, and generates free radicals [41]. All of these interact with NO. Moreover, prolyl hydroxylase is inhibited by poly(ADP ribose) [42], the synthesis of which is stimulated by NO [43]. Further work is required to confirm that NO inhibits this enzyme and to determine the mechanism of inhibition. It should be noted, however, that the data suggest that NO-independent processes predominate in down-regulating prolyl hydroxylase activity.

If inhibition of prolyl hydroxylase is indeed the mechanism of action of NO, then this radical should inhibit the synthesis of not only types-II and -V/XI collagen, but other collagens too. Indeed, there are preliminary data that this might be so [34]. In this way, NO could function as a novel anti-fibrotic agent with wide clinical application. There is already considerable interest, in this regard, in the use of NO to prevent restenosis after balloon angioplasty [44].

The ability of NO to inhibit the biosynthesis of both of the major macromolecular constituents of the cartilaginous matrix make it a potentially powerful agent of cartilage pathology in arthritis and other joint disorders.

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