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Inhibition of Inducible Nitric Oxide Synthase Prevents Lipid Peroxidation in Osteoarthritic Chondrocytes

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ABSTRACT

Nitric oxide (NO) and the lipid peroxidation (LPO) product 4-hydroxynonenal (HNE) are considered to be key mediators of cartilage destruction in osteoarthritis (OA). NO is also known to be an important intermediary in LPO initiation through peroxynitrite formation. The aim of the present study was to assess the ability of the inducible NO synthase (iNOS) inhibitor N-iminoethyl-L-lysine (L-NIL) to prevent HNE generation via NO suppression in human OA chondrocytes and cartilage explants. Human OA chondrocytes and cartilage explants were treated with L-NIL and thereafter with or without interleukin-1beta (IL-1β) or HNE at cytotoxic or non-cytotoxic concentrations. Parameters related to oxidative stress, apoptosis, inflammation, and catabolism were investigated. L-NIL stifled IL-1β-induced NO release, iNOS activity, nitrated proteins, and HNE generation in a dose-dependent manner. It also blocked IL-1β-induced inactivation of the HNE-metabolizing glutathione-s-transferase (GST). L-NIL restored both HNE and GSTA4-4 levels in OA cartilage explants. Interestingly, it also abolished IL-1β-evoked reactive oxygen species (ROS) generation and p47 NADPH oxidase activation. Furthermore, L-NIL significantly attenuated cell death and markers of apoptosis elicited by exposure to a cytotoxic dose of HNE as well as the release of prostaglandin E_2 and metalloproteinase-13 induced by a non-cytotoxic dose of HNE. Altogether, our findings support a beneficial effect of L-NIL in OA by (i) preventing the LPO process and ROS production via NO-dependent and/or independent mechanisms and (ii) attenuating HNE-induced cell death and different mediators of cartilage damage. J. Cell. Biochem. 113: 2256–2267, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: NITRIC OXIDE; LIPID PEROXIDATION; 4-HYDROXYNONENAL; OSTEOARTHRITIS; CARTILAGE; GLUTATHIONE-S-TRANSFERASE

Nitric oxide (NO) is a free radical that undergoes a variety of reactions in tissues with other radicals, such as superoxide or molecular oxygen, leading to the generation of highly damaging nitrosating species [Squadrito and Pryor, 1998]. It has also been shown to cause nitrosation or oxidation of zinc finger-containing proteins involved in DNA repair [Sidorkina et al., 2003]. Furthermore, in vitro and in vivo cell exposure to NO from donor drugs can modulate the activity of mitochondrial enzymes, metalloproteinases (MMPs) and protein kinases via a nitrosylation process [Gu et al., 2002, 2010]. NO overproduction damages cellular components, including lipids, which results in declining physiological function and cell death. NO reaction with lipids generates lipid peroxidation (LPO), giving rise to 4-hydroxynonenal (HNE) [Morquette et al., 2006]. LPO initiation by NO in biological systems requires the generation of superoxide anion and hydroxyl radicals [Carrico et al., 2009; Szabo and Modis, 2010]. A mechanism of hydroxyl radical production, which has been proposed, involves the reaction of superoxide radical with NO, forming peroxynitrite. Experiments with pure peroxynitrite have shown that it can decompose spontaneously into hydroxyl radicals and initiate the oxidative modification of both lipids and proteins. In vivo, the main sources of NO and anion superoxide remain inducible NO synthase (iNOS) and NADPH oxidase (NOX), respectively [Montezano and Touyz, 2012]. Consistent with these data, the ability of peroxynitrite to evoke LPO necessitates perfect coordination between iNOS and NOX expression and activity. Thus, the development of inhibitors of both enzymes could have substantial benefits in the treatment of various disease conditions, including osteoarthritis (OA), in which oxidative stress is increased.

There is ample evidence that NO plays a key role in OA pathogenesis. Upon exposure to pro-inflammatory cytokines, OA cartilage produces excessive amounts of iNOS protein and its end

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product NO [Amin et al., 1995; Castro et al., 2006]. High levels of nitrite/nitrate also have been found in synovial tissue from OA patients [McInnes et al., 1996]. It has been hypothesized that NO contributes to the development of arthritic lesions by inhibiting the synthesis of cartilage matrix macromolecules [Amin and Abramson, 1998] and by inducing chondrocyte death [Nakagawa et al., 2010], which could further contribute to extracellular matrix (ECM) reduction in OA. For example, anterior cruciate ligament transection in rabbits provokes chondrocyte apoptosis and NO production, events that can, however, be prevented by treatment with iNOS inhibitors [Hashimoto et al., 1998]. The suppression of NO generation also significantly curbs interleukin-1beta (IL-1β)-induced chondrocyte apoptosis [Chen et al., 2006] and reduces the progression of cartilage damage in experimental OA model in dog [Pelletier et al., 1998].

Like NO and reactive oxygen species (ROS), aldehydes are electrophiles that bind to nucleophilic groups of proteins, but their relatively longer half-lives make them candidates for the propagation of damage to neighbouring cells [Uchida, 2003]. Aldehyde levels increase significantly under intense oxidative stress, and this elevation is believed to contribute to the development of many pathological conditions [Poli et al., 2008]. HNE, the principal α,β-unsaturated aldehyde formed from the LPO of both ω-3 and ω-6 polyunsaturated fatty acids, is of specific interest in the present study as its formation is enhanced in synovial fluid from OA patients [Morquette et al., 2006]. In addition, growing evidence supports its role as a key modulator of catabolic and inflammatory mediators known for their involvement in the OA process [Morquette et al., 2006; Vaillancourt et al., 2008; El-Bikai et al., 2010]. The electrophilic nature of HNE makes it highly reactive with specific amino acids in proteins via Michael-type addition: The sulfhydryl group of cysteine (CYS) or lipoic acid, the ε-amino group of lysine (LYS), or the imidazole function of histidine (HIS) [Esterbauer et al., 1991; Wakita et al., 2011]. HNE homeostasis depends primarily on many factors, in addition to the magnitude of free radical production. Thus, conjugation to glutathione (GSH) by glutathione-s-transferase A4-4 (GSTA4-4) is a major route of HNE elimination [Balogh and Atkins, 2011]. It has been shown that GSTA4-4 metabolizes HNE with high catalytic efficiency through its conjugation to GSH and has been suggested to be a major component of cellular defence against HNE toxicity [Vaillancourt et al., 2008; Sharma et al., 2011].

While investigating the potential regulation of HNE levels in OA cartilage, we obtained data indicating that its production is regulated by NO generation. In this study, we tested the hypothesis that suppression of NO release with the iNOS inhibitor N-iminoethyl-L-lysine (L-NIL) is a novel strategy to abrogate the LPO process and HNE generation in OA.

MATERIALS AND METHODS

SPECIMEN SELECTION AND CHONDROCYTE CULTURE

Discarded human post-surgery OA articular cartilage was obtained from OA patients ($n = 21$, age 67 ± 9 years mean $\pm$ SEM) who underwent total knee arthroplasty. Informed consent was received from them to use their tissues for research purposes. All patients were evaluated by rheumatologists who followed American College of Rheumatology criteria [Altman et al., 1986]. The experimental protocols and research into human tissues were approved by the Research Ethics Board of Hôpital du Sacré-Cœur de Montréal.

OA knee cartilage specimens were sliced and rinsed before chondrocyte extraction by sequential enzymatic digestion, as described previously [Morquette et al., 2006]. Cartilage samples were digested with 1 mg/ml of pronase (Sigma-Aldrich, Oakville, ON) for 1 h at 37°C, and then with 2 mg/ml of type IV collagenase (Sigma-Aldrich) for 6 h in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen). Chondrocytes were seeded at high density in culture flasks at 37°C in a humidified atmosphere of 5% CO_2/95% air until they were confluent and ready for experimentation.

EXPERIMENTAL CULTURE CONDITIONS

OA chondrocytes (2×10^5 cells/cm^2) were pre-treated with increasing concentrations of L-NIL (0–20 μM, Pfizer Canada Inc., Kirkland, QC) for 1 h, followed by incubation with or without either 1 ng/ml of IL-1β (Sigma-Aldrich), 10 μM 3-morpholinosydnonimine (SIN, Sigma-Aldrich), or cytotoxic (20 μM) or non-cytotoxic (10 μM) doses of HNE (Cayman Chemical, Ann Arbor, MI) for 24 h in 1% FBS-DMEM.

PROSTAGLANDIN E2 (PGE_2), METALLOPROTEINASE-13 (MMP-13), AND NO DETERMINATION

After chondrocyte incubation for 24 h, the medium was collected, and PGE_2 and MMP-13 levels were assessed by enzyme immunoassay (Cayman Chemical) and with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), respectively. Detection sensitivity was 9 and 8 pg/ml, respectively. All assays were performed in duplicate. Nitrite, a stable end-product of NO, was quantified in the supernatant according to a spectrophotometric method based on Griess reaction [Green et al., 1982]. Absorbance was measured with a micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT).

CELL VIABILITY

Chondrocyte viability was evaluated, as described previously [Mosmann, 1983], by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay in 96-well plates (Fisher Scientific Company, Ottawa, ON) by incubating the cells with 0.5 mg/ml MTT reagent (Sigma-Aldrich) for 15 min at 37°C. Then, 100 μl of solubilization solution (0.04 M HCl–isopropanol) was added, formazan salt was dissolved, and absorbance was read at 570 nm with the micro-ELISA Vmax photometer (Bio-Tek Instruments).

iNOS ACTIVITY ASSAY

Culture supernatants were removed and the cells were washed three times with phosphate-buffered saline (PBS) and lysed by incubation with lysis buffer (40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM

NaF, 0.3 mM Na_3VO_4, 1 mM dithiothreitol) supplemented with protease inhibitor cocktail. Protein concentration of the cell lysates was measured by Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON). iNOS activity was assessed, as described previously [Sosroseno et al., 2011]. Briefly, 25 μg of chondrocyte lysate were incubated for 2 h at 37°C in 100 μl of 20 mM Tris-HCl (pH 7.9) containing 5 μM tetrahydrobiopterin (BH_4), 5 μM FAD, 1 mM dithiothreitol, 1 mM NADPH, and 1 mM L-arginine. All materials were purchased from Sigma-Aldrich. The reaction was then stopped by adding lactate dehydrogenase (10 U/ml), and NO levels were measured by the Griess method described above. Each assessment was repeated three times.

PROTEIN DETECTION BY WESTERN BLOTTING

20 μg of total proteins of chondrocyte lysates, treated under the indicated conditions, were loaded for discontinuous 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories) for protein immunodetection and semi-quantitative measurement [Morquette et al., 2006]. The primary antibodies deployed were rabbit anti-human NOX component p47phox (p47 NOX, Cell Signalling Technology, Inc., Danvers, MA), anti-HNE (Cayman Chemical), anti-nitrotyrosine (Cayman Chemical), anti-caspase-3 (EMD Biosciences, Inc., San Diego, CA), and anti-human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). After serial washes, the primary antibodies were revealed by goat anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Cell Signalling Technology, Inc.). Immunoreactive proteins were detected with SuperSignal blotting substrate (Pierce, Rockford, IL) and exposed to Kodak X-Omat film (Eastman Kodak Company, Rochester, NY).

CELLULAR LEVEL OF HNE–PROTEIN ADDUCTS

Total cellular levels of HNE-protein adducts were calculated in chondrocyte extracts under the indicated conditions by in-house ELISA [Morquette et al., 2006]. HNE-modified bovine serum albumin (BSA) served as standard for HNE-protein adduct assay.

IMMUNOHISTOCHEMISTRY

Human OA cartilage explants (~150 mg) were incubated for 1 h in the presence or absence of 10 mM L-NIL, followed by another incubation for 48 h in the presence of 1 ng/ml IL-1β. The cartilage specimens were fixed in TissuFix #2 (Laboratoires Gilles Chaput, Montreal, QC) for 24 h, then embedded in paraffin. Sections (5 μm) of paraffin-embedded specimens were de-paraffinized in toluene, rehydrated in a reverse-graded series of ethanol and pre-incubated with 0.25 units/ml chondroitinase ABC (Sigma-Aldrich) in PBS (pH 8.0) for 60 min at 37°C. The sections were subsequently washed in PBS, incubated in 0.3% Triton X-100 for 20 min, then placed in 3% hydrogen peroxide/PBS for 15 min. The slides were further incubated with blocking serum (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) for 60 min, after which they were blotted and overlaid with the primary antibody against HNE (1/200 dilution, EMD Chemicals, Inc. Gibbstown) or GSTA4-4 (1/500 dilution, Sigma-Aldrich) for 18 h at 4°C in a humidified chamber.

Each slide was washed three times in PBS (pH 7.4), stained by the avidin–biotin complex method (Vectastain ABC kit), and incubated in the presence of biotin-conjugated secondary antibody for 45 min at room temperature, followed by the addition of avidin–biotin–peroxidase complex for 45 min. All incubations were undertaken in a humidified chamber at room temperature, and staining was developed with 3,3'-diaminobenzidine (DAKO Diagnostics Canada Inc., Mississauga, ON) containing hydrogen peroxide. The slides were counterstained with hematoxylin/eosin.

The sections were examined and photographed with a light Leica DM IRB microscope (Leica, Heerbrugg, Switzerland) equipped with a Leica DFC425 C camera (Leica). The presence of the antigen in cartilage was quantified by determining the number of chondrocytes that stained positive throughout cartilage thickness. Three sections from cartilage explants were examined, and the results were the means of positive chondrocytes counted in three light microscopic fields. The total number of chondrocytes and those staining positive in each field for the specific antigen were quantified. The final results were expressed as percentages of chondrocytes staining positive for the antigen (cell score), with the maximum score being 100%. Each slide was viewed by two independent readers who were blinded to treatment group allocation.

ROS MEASUREMENT

Intracellular ROS formation was measured with MitoSOX™ Red reagent (Invitrogen). Oxidation of MitoSOX™ Red reagent by superoxide produces red fluorescence, as described by Zhou et al. [2011]. Briefly, chondrocytes were seeded at a density of 2×10^4 cells/well in 96-well black plates (Becton Dickinson, San Jose, CA). They were pre-treated with increasing concentrations of L-NIL 1 h before their exposure to IL-1β. Fluorescence was measured with a fluorescence plate reader at 510-nm absorption and 580-nm emission, and the data were expressed as relative MitoSOX™ Red fluorescence. To detect ROS in cells, the chondrocytes were transferred to 8-well culture chamber slides (Lab-Tek, Nalge Nunc International, Naperville, IL), and incubated with MitoSOX™ Red reagent and 4′,6-Diamidino-2-phenylindole (DAPI). ROS generation was then measured every 30 s for 15 min with a Leica DM IRB fluorescence microscope (Leica) equipped with a Leica DFC425 C camera (Leica).

TOTAL GST ACTIVITY

Total GST activity was assessed with a commercial kit (Sigma-Aldrich) in 50 μg of chondrocyte extract. Briefly, the cells were homogenized on ice in 1 ml of buffer containing 180 mM KCl, 5 mM MOPS, and 2 mM EDTA, pH 7, and centrifuged for 10 min at 800*g* at 4°C. The supernatants were subjected to enzyme assays after 10-min centrifugation at 6,000*g* at 4°C. Protein levels were measured with a kit (Bio-Rad Laboratories) with BSA (Sigma-Aldrich) as standard. Activities were expressed in units/mg of proteins, where 1 unit was defined as the amount of enzyme catalyzing the conversion of 1 μmol substrate/min at 37°C.

DNA FRAGMENTATION

Cytoplasmic histone-associated DNA fragments were quantified with Cell Death Detection ELISA^{PLUS} kits (Roche Applied Science,

Laval, QC) according to the manufacturer's recommendations. Briefly, cultured chondrocytes (1×10^6 cells) were lysed with lysis buffer for 30 min and centrifuged at 200*g* for 10 min. The supernatant and a mixture of anti-histone-biotin and anti-DNA-peroxidase were added to streptavidin-coated microplates and incubated for 2 h at room temperature. Absorbance was measured at 405 nm after inclusion of the substrate.

STATISTICAL ANALYSIS

All values are expressed as means ± SEM unless indicated otherwise. Multiple comparisons were made by 1-way ANOVA, as required, followed by the Bonferroni multiple-comparison post-test. Statistical comparisons were performed with GraphPad Prism software, version 4b (GraphPad Software, San Diego, CA). In all tests, the criterion for statistical significance was $P < 0.05$.

RESULTS

L-NIL PREVENTS NO AND iNOS PRODUCTION IN OA CHONDROCYTES

In our first objective, we performed initial experiments to demonstrate that L-NIL prevents NO release and inhibits iNOS activity in isolated human OA chondrocytes. Cells were treated for 1 h with increasing doses of L-NIL (0–20 μM) and thereafter with or without 1 ng/ml IL-1β for 24 h. The addition of L-NIL significantly reduced IL-1β-induced NO release (Fig. 1A) and iNOS activity (Fig. 1B) in a dose-dependent manner. At 10 μM, L-NIL suppressed NO release by 80% ($P < 0.001$) and iNOS activity by 90% ($P < 0.001$). Collectively, these data confirm that L-NIL is a potent iNOS inhibitor.

L-NIL BLOCKS HNE PRODUCTION THROUGH NO AND PEROXYNITRITE INHIBITION

We and other research groups have reported the ability of NO to induce LPO via peroxynitrite generation [Morquette et al., 2006]. In the present study, we tested the hypothesis that NO inhibition suppresses peroxynitrite and, consequently, the production of HNE, a very reactive product of LPO. As illustrated in Figure 2A, the addition of 1 ng/ml IL-1β or 10 μM SIN, a donor of free radicals, induced a similar pattern of both NO release and HNE-protein adducts production. Compared to untreated cells, NO levels reached 15 ± 2.5 nmol/2×10^5 cells ($P < 0.01$) and 12 ± 3 nmol/2×10^5 cells ($P < 0.05$), and HNE attained 600 ± 55 and 650 ± 86 pg/mg proteins ($P < 0.01$) in the presence of SIN or IL-1β, respectively. Before testing the ability of L-NIL to inhibit HNE production, we investigated whether this drug blocks peroxynitrite generation. As expected, western blotting revealed that nitrotyrosine levels in nitrated proteins were lower in L-NIL-treated cells than in IL-β-treated cells (Fig. 2B). Interestingly, the addition of L-NIL at different dose levels also abolished IL-1β-induced HNE production. Our ELISA and western blotting data (Fig. 2C,D) disclosed that L-NIL, at 10 μM, prevented HNE generation ($P < 0.01$) in cell extracts from IL-1β-treated OA chondrocytes. Afterwards, we tested the ability of L-NIL to prevent (or not) the IL-1β-induced inactivation of HNE-metabolizing GST. As illustrated in Figure 2E, L-NIL addition to

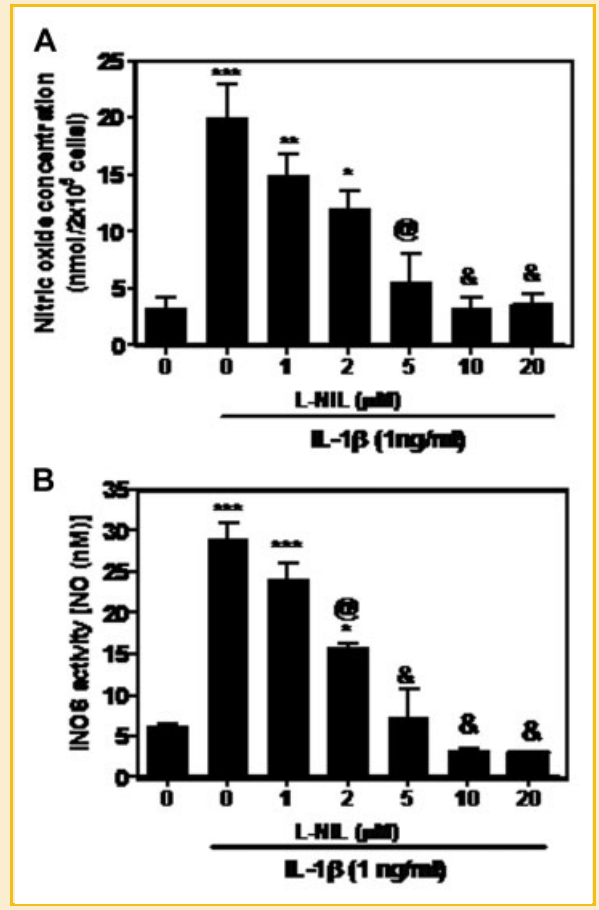

Fig. 1. N-iminoethyl-L-lysine (L-NIL) reduces nitric oxide (NO) release and inducible nitric oxide synthase (iNOS) activity. Human osteoarthritis (OA) chondrocytes were treated with increasing L-NIL concentrations (0–20 μM) for 1 h before incubation for 24 h in the presence of 1 ng/ml interleukin-1beta (IL-1β). A: NO release was assessed by the improved Griess method. B: iNOS activity was measured in the presence of arginine, BH_4, and NADPH. Values represent the means ± SEM of 3–4 separate experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells (control) (1% FBS); $^{@}P < 0.01$, $^{\&}P < 0.001$ compared to IL-1β-treated cells.

cultured cells blocked GST inactivation by IL-1β ($P < 0.05$). Taken together, these findings strongly suggest that HNE production is linked, at least in part, to NO release and iNOS activation. L-NIL could be considered an interesting tool to abrogate both NO generation and LPO in OA chondrocytes. It also prevented impairment of redox status in IL-1β-treated cells.

IMMUNOHISTOCHEMISTRY OF HNE AND GSTA4-4

Chondrocytes staining positive for HNE and GSTA4-4 were found in OA cartilage. Compared to the controls (Fig. 3A), chondrocytes stained positive for increased HNE, which reached 61% (Fig. 3B, $P < 0.01$) in IL-1β-treated OA cartilage. However, 10 μM L-NIL significantly prevented IL-1β-induced HNE production in OA cartilage (Fig. 3C, $P < 0.05$). Furthermore, compared to the controls (Fig. 3D), chondrocytes stained positive for decreased GSTA4-4, which reached 14% (Fig. 3E, $P < 0.01$) in IL-1β-treated OA cartilage.

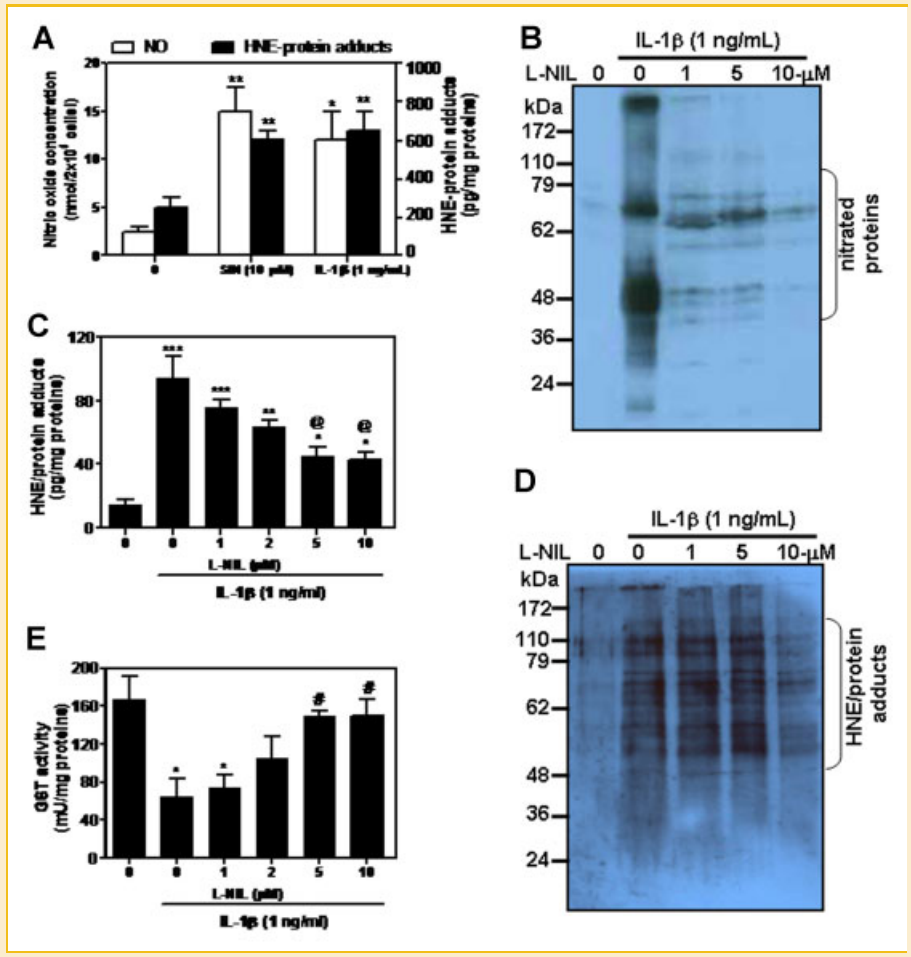

Fig. 2. N-iminoethyl-l-lysine (l-NIL) suppresses the lipid peroxidation product 4-hydroxynonenal (HNE). A: Confluent human osteoarthritic (OA) chondrocytes were treated with either 10 μM SIN (a NO donor) or 1 ng/ml IL-1β for 24 h. NO and HNE generation was assessed by the improved Griess method or ELISA, respectively. (B–E) Human OA chondrocytes were treated with increasing doses of L-NIL (0–10 μM) for 1 h, followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. The cells were then lysed and analyzed. Nitrated protein levels were evaluated by western blotting (B), HNE/protein adducts were quantified by ELISA (C) and analyzed by western blotting (D), and GST activity was assessed with a commercial kit (E). The NO, HNE/protein adduct and GST activity results are expressed as nmol/2×10^5 cells, pg/mg proteins or mU/mg proteins, respectively, and represent the means ± SEM of four independent experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells (1% FBS); $^{\#}P < 0.05$, $^{@}P < 0.01$ compared to IL-1β-treated cells alone. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

However, 10 μM L-NIL significantly abolished IL-1β inhibition of GSTA4-4 expression in OA cartilage (Fig. 3F, $P < 0.05$). Altogether, our results confirm that the iNOS inhibitor L-NIL attenuates oxidative stress and restores redox status by reducing HNE generation and reinstating GSTA4-4 expression in cartilage explants.

L-NIL ABOLISHES ROS GENERATION AND P47 NOX PHOSPHORYLATION

It is well documented that the generation of ROS, such as superoxide anion and hydroxyl radical, plays an important role in initiating LPO. Thus, the purpose of this part of the present study was to investigate whether L-NIL's capability to inhibit HNE production is attributed to its ability to also prevent ROS generation. To do so, OA chondrocytes were pre-treated with 10 μM L-NIL for 1 h, followed by exposure to 1 ng/ml IL-1β for 24 h. ROS generation was observed by fluorescence microscopy and then quantified with MitoSOX™ Red reagent. As illustrated in Figure 4B, elevated ROS levels were detected by fluorescence microscopy in OA chondrocytes treated with 1 ng/ml IL-1β compared to untreated cells (Fig. 4A). Interestingly, ROS production was quenched by L-NIL treatment (Fig. 4C). DAPI staining, which reveals nuclei, was employed to assess their number and to examine gross cell morphology. Quantitatively, our data indicated that relative MitoSOX™ Red fluorescence was higher in 1 ng/ml IL-1β-treated cells and reached 220 ± 36 ($P < 0.05$) (Fig. 4D). However, when the cells were treated with both 1 ng/ml IL-1β and 10 μM L-NIL, relative MitoSOX™ Red fluorescence decreased significantly to 121 ± 16 ($P < 0.05$) (Fig. 4D). Finally, an additional experiment was performed to determine the possible ability of L-NIL to inhibit p47 NOX phosphorylation, a ROS-generating enzyme. Western blotting analysis showed that 10 μM L-NIL prevented IL-1β-induced p47 NOX phosphorylation (Fig. 4E). Collectively, our data indicated that suppression of ROS production and ROS-generating NOX by the selective inhibitor of iNOS had a significant LPO outcome.

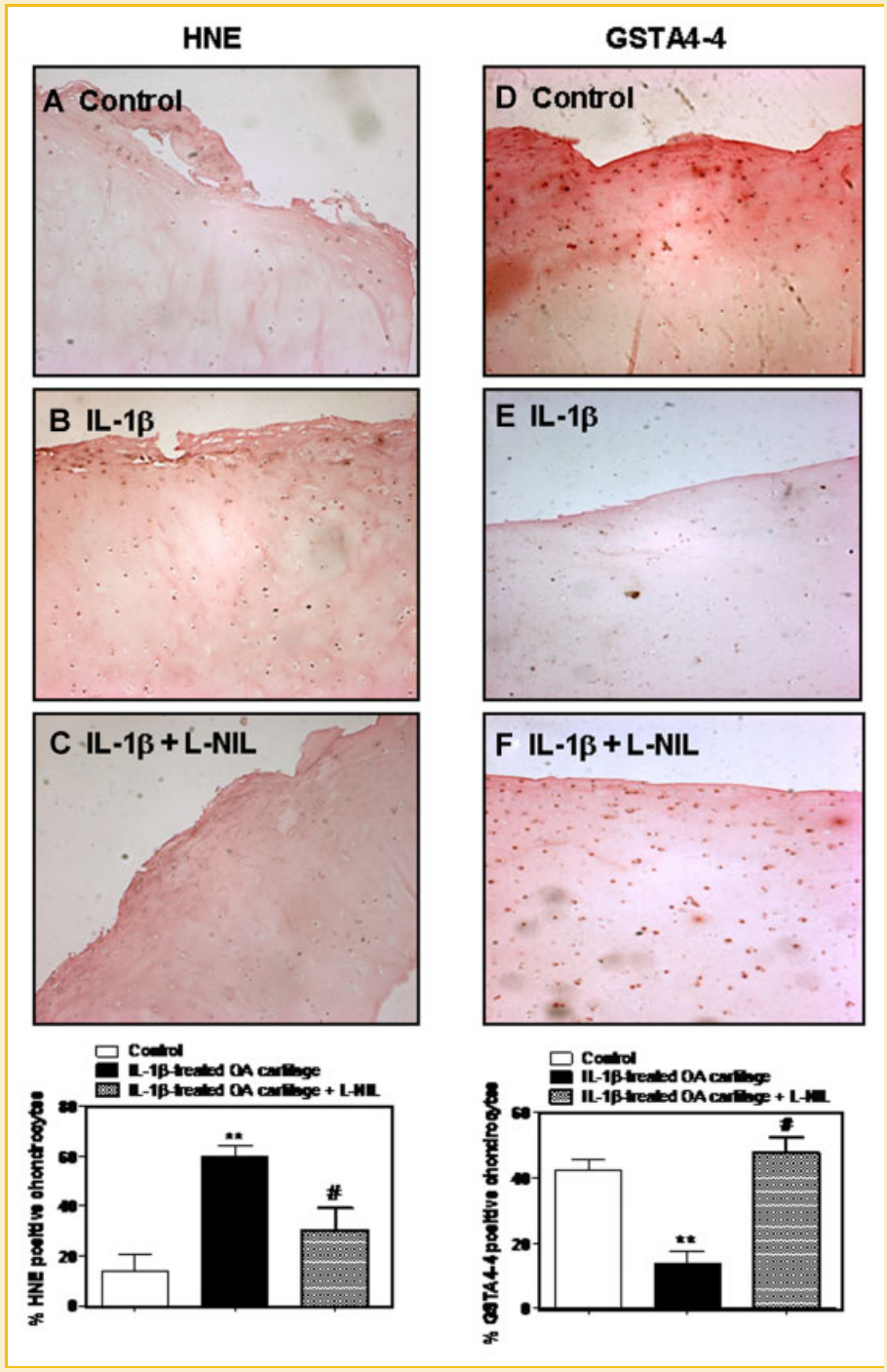

Fig. 3. Immunohistochemistry of HNE and GSTA4-4. Representative section showing 4-hydroxynonenal (HNE) (A–C) and GSTA4-4 (D–F) immunostaining in superficial zones of articular cartilage from human osteoarthritis (OA) cartilage explants treated or not for 1 h with 10 μM L-NIL, followed by another incubation for 24 h with 1 ng/ml IL-1β (original magnification ×100). The number of chondrocytes staining positive for HNE and GSTA4-4 were evaluated and analyzed as described in Materials and Methods section. $^{**}P < 0.01$ compared to untreated OA cartilage (control); $^{\#}P < 0.05$ compared to IL-1β-treated cells.

HNE-INDUCED CELL DEATH IS BLOCKED BY L-NIL

We reported that HNE up to 10 μM did not alter cell viability, but 20 μM HNE was cytotoxic and significantly decreased cell viability by approximately 50% [Vaillancourt et al., 2008]. We evaluated the ability of L-NIL to reduce HNE cytotoxicity in cultured chondrocytes. Cell viability was assessed with MMT reagent. After 24 h of incubation, pre-treatment with 5 and 10 μM L-NIL for 1 h before adding 20 μM HNE to the culture media prevented HNE-induced cell death (Fig. 5A) as well as markers of apoptosis, including caspase-3 activation (Fig. 5B) and DNA fragmentation (Fig. 5C). These data suggest that L-NIL probably prevents HNE's effects through direct HNE quenching.

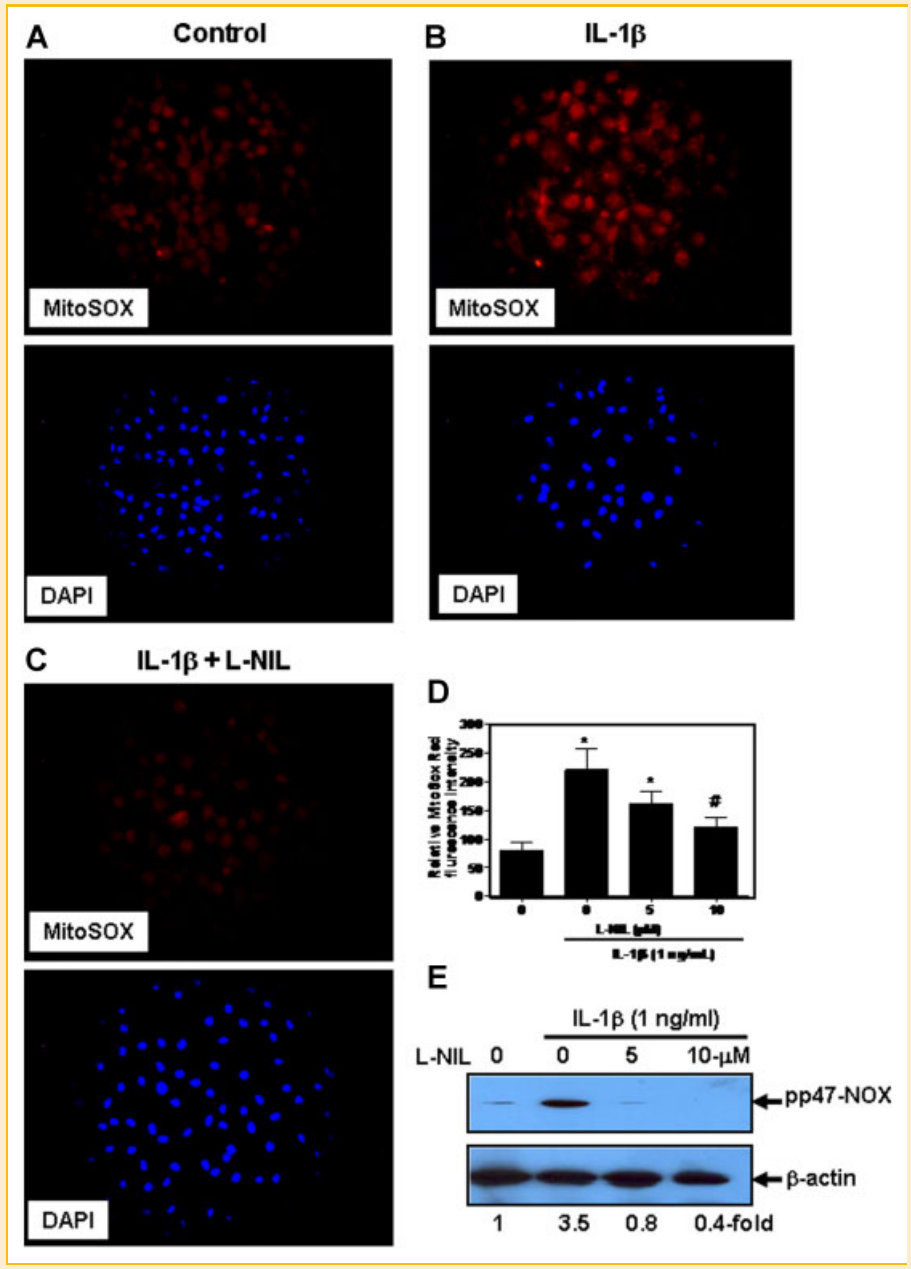

Fig. 4. N-iminoethyl-l-lysine (L-NIL) suppresses reactive oxygen species (ROS) generation and NADPH oxydase (NOX) phosphorylation. (A–C) Confluent human osteoarthritic (OA) chondrocytes were treated for 1 h with 10 μM L-NIL, followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. ROS generation was revealed by fluorescence microscopy with MitoSOX™ Red reagent. D, E: Cells were treated for 1 h with increasing concentrations of L-NIL (0–10 μM), followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. D: ROS generation was quantified in cell extracts with MitoSOX™ Red reagent. ROS levels were expressed as relative MitoSOX™ Red fluorescence. E: p47 NOX protein phosphorylation (pp47 NOX) was analyzed by western blotting in protein extracts from human OA chondrocytes treated as described above. The data are means ± SEM of four independent experiments. $^{*}P < 0.05$ compared to untreated cells (control) (1% FBS); $^{\#}P < 0.05$ compared to IL-1β-treated cells alone.

HNE-INDUCED PGE_2 AND MMP-13 PRODUCTION ARE ABROGATED BY L-NIL

This part of our study was designed to verify L-NIL's ability to attenuate HNE-evoked the production of inflammatory and catabolic mediators known to be involved in cartilage damage, such as PGE_2 and MMP-13, respectively. When the cells were treated with non-cytotoxic doses of HNE (at 10 μM) [Vaillancourt et al., 2008], L-NIL prevented HNE-induced PGE_2 (Fig. 6A) and MMP-13 (Fig. 6B) release. At 10 μM, L-NIL reduced PGE_2 and MMP-13 levels by 55 ($P < 0.01$) and 60% ($P < 0.05$), respectively.

DISCUSSION

Articular cartilage is believed to be a major source of NO in OA joints and synovial fluid [Pelletier et al., 1998]. Previous in vitro studies

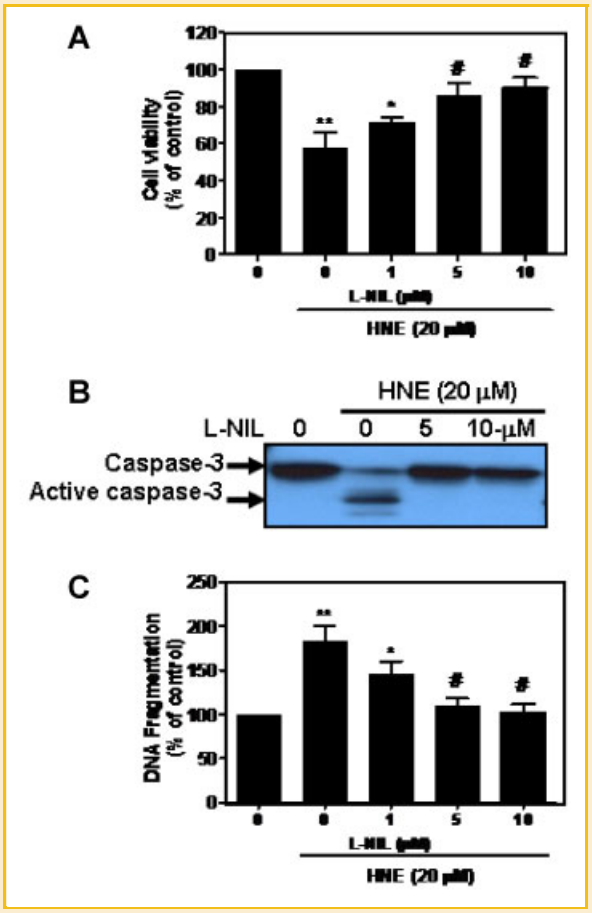

Fig. 5. 4-Hydroxynonenal (HNE)-induced cell death is blocked by L-NIL. Chondrocytes were pre-incubated for 1 h with or without increasing doses of L-NIL (0–10 μM) followed by another incubation for 24 h with a cytototoxic HNE dose (20 μM). A: Cell viability was evaluated by MTT assay. B: Activation of caspase-3 was determined by western blotting. C: Cytoplasmic histone-associated DNA fragments were quantified with a kit. The data, expressed as % of untreated cells for cell viability and DNA fragmentation, are the means ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to untreated cells (1% FBS); $^{\#}P < 0.05$ compared to HNE-treated cells alone. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

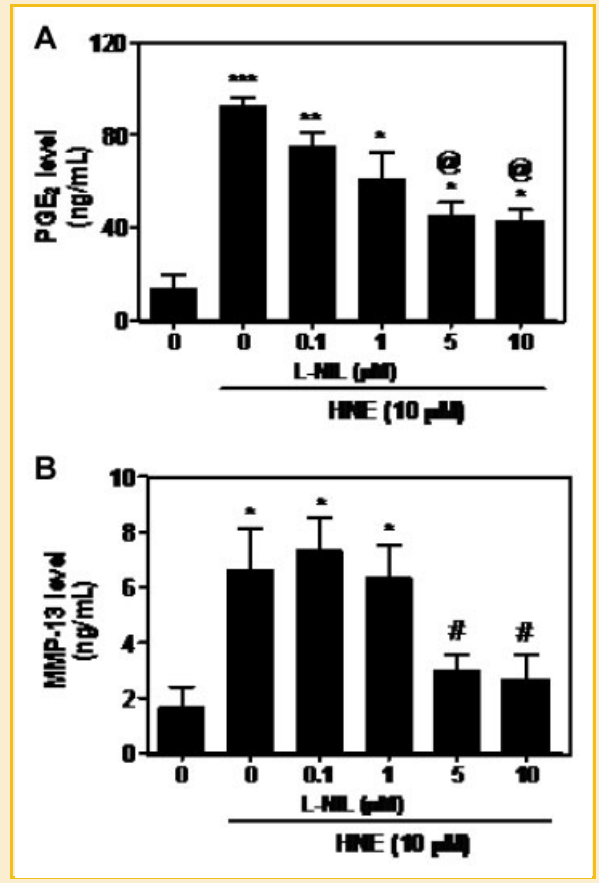

Fig. 6. N-iminoethyl-L-lysine (L-NIL) prevents 4-hydroxynonenal (HNE) induction of prostaglandin E2 (PGE_2) and metalloproteinase-13 (MMP-13). Human osteoarthritic (OA) chondrocytes were treated for 1 h with different doses of L-NIL (1–10 μM) before incubation with non-cytotoxic (10 μM) doses of HNE for 24 h. Supernatants were collected, and PGE_2 (A) and MMP-13 (B) levels were measured with commercial enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) kits, respectively. The data, expressed as ng/ml for PGE_2 and MMP-13 protein, represent the means ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells (1% FBS); $^{\#}P < 0.05$, $^{@}P < 0.01$ compared to HNE-treated cells alone.

have shown that chondrocytes produce NO as a key metabolite in response to stimuli such as mechanical stress, IL-1α, IL-1β, and lipopolysaccharides (LPS) [Abramson, 2008]. In addition, iNOS is up-regulated in OA chondrocytes, resulting in excess NO and perpetuating the release of inflammatory cytokines and other catabolic processes. NO inhibits both proteoglycan and collagen synthesis, activates MMPs, mediates chondrocyte apoptosis, and promotes chondrocyte inflammatory responses [Abramson, 2008]. These findings add to the prevailing hypothesis that NO is a pro-inflammatory and pro-apoptotic factor that, when present in excess, is detrimental to the joints and contributes to OA pathogenesis. A number of iNOS inhibitors have been developed to prevent NO production in OA joint tissues. Among them, L-NIL is a potent and selective iNOS inhibitor. However, the detrimental effects of NO in OA cartilage have been challenged by a number of reports showing that NO may inhibit pro-inflammatory responses by preventing nuclear factor-kappa B (NF-κB) transactivation in cultured chondrocytes [Rosa et al., 2008]. In addition, under some conditions, exogenous NO can stimulate collagen synthesis in cultured human tendon cells [Xia et al., 2006]. In vivo, Veihelmann et al. [2001] demonstrated that NO production by iNOS has anti-inflammatory effects in experimental arthritis, by mediating a reduction of leukocyte adhesion and infiltration. Further research may help to elucidate a potential role for NO-donating agents in OA management.

In the present study, our observations provided the impetus for a novel therapeutic target of the iNOS inhibitor L-NIL, namely, ROS and the LPO product HNE, in OA. In our in vitro experiments, NO generation and oxidative stress were induced in human OA chondrocytes treated with IL-1β or SIN (a NO donor). First, we obtained data showing that L-NIL significantly reduced both IL-β-evoked NO and peroxynitrite release as well as iNOS activity in

human OA chondrocytes. These findings are in agreement with numerous reports indicating that this drug is a potent inhibitor of NO production via iNOS inactivation in OA in vitro and in vivo. In cartilage explants, L-NIL markedly curbed chondrocyte apoptosis by obviating NO generation [Pelletier et al., 2001]. In an experimental dog model of OA, oral administration of L-NIL decreased NO production, resulting in marked reductions of major catabolic factors, such as MMPs, IL-1β, and peroxynitrite, as well as diminution of cyclooxygenase-2 (COX-2) expression [Pelletier et al., 1999]. Macroscopically, L-NIL diminished cartilage lesion size by approximately 50% on both condyles and plateaus. The histological severity of both cartilage lesions and synovial inflammation was significantly curtailed in L-NIL-treated dogs [Pelletier et al., 1998]. However, these data contrast with those reported by McCartney-Francis et al. [2001] who demonstrated that L-NIL administration exacerbated the chronic inflammatory response, as reflected by profound tissue destruction and bone and cartilage losses. Altogether, such findings warrant caution as to the possible utility of NOS inhibitors in OA therapy, necessitating enhanced knowledge and understanding of the (patho)physiological role of iNOS in OA.

Second, recognizing the important role of LPO products in OA, we tested the hypothesis that inhibition of NO generation in OA chondrocytes culminates in the prevention of HNE generation, a very reactive LPO product. Our findings revealed, for the first time, that L-NIL blocked IL-1β-induced HNE-protein adduct accumulation in isolated chondrocytes and cartilage explants, indicating that NO is involved in LPO. It has been established that NO can promote LPO via peroxynitrite generation. Over the last few years, reactive LPO products have been at the center of the pathophysiological scene of OA disease. In a previous report, we documented a significant increase of endogenous HNE levels in its adducted form in synovial fluid samples from OA patients compared to control subjects [Morquette et al., 2006]. These data are consistent with the findings of Grigolo et al. [2003] and Shah et al. [2005], who observed higher levels of both malondialdehyde and HNE in human articular tissues from OA patients in comparison to healthy subjects. In addition, incubation of isolated OA chondrocytes with tumour necrosis factor-alpha or NO donors (e.g., SIN) provided direct evidence implicating these mediators in HNE production, given that all of them were able to increase HNE/protein adduct levels in cellular extracts [Morquette et al., 2006]. Like NO, HNE has the ability to activate a panoply of factors known for their involvement in OA. For example, in OA cartilage, HNE can provoke transcriptional as well as post-translational modifications of collagen type II (Col II) and MMP-13, resulting in cartilage ECM degradation [Morquette et al., 2006]. In addition, HNE can selectively stimulate COX-2 expression via activating transcription factor/cAMP response element and inhibit iNOS expression via NF-κB inactivation in human chondrocytes [Vaillancourt et al., 2007]. More recently, we demonstrated that HNE-binding to Col II culminates in multiple abnormalities in chondrocyte phenotype and function, indicating its contribution to alteration of cell-ECM interaction in OA [El-Bikai et al., 2010]. Taken together, and in conjunction with the fact that L-NIL abolishes HNE generation and restores redox status, these findings open new avenues for this drug in the treatment of OA patients.

To determine the impact of L-NIL treatment on HNE-metabolizing enzymes in chondrocytes, we conducted additional experiments to verify its effect on GST activity in chondrocytes. As predicted, our data showed that L-NIL prevented IL-1β-induced GST inactivation, suggesting normalization of redox status by its administration. Our results are in agreement with other observations indicating that pro-inflammatory cytokines and LPS inhibit GST expression. The transcriptional suppression of GST by IL-1β is known to be mediated by hepatic nuclear factor [Ng et al., 2007]. Furthermore, our findings strongly suggest that GST inactivation after IL-1β addition could be attributed, in part, to NO generation. The decrease in total GST activity in cartilage can compromise tissue defence against HNE and other electrophiles. Several lines of evidence have revealed that GST is a target of nitrosylation and oxidation by NO and ROS, respectively [Cesareo et al., 2005; Letelier et al., 2010]. While multiple GST isoforms exist with distinct catalytic properties, structural observations have indicated that most cytosolic GST isoforms contain a highly-conserved tyrosine (TYR) residue which is crucial for their catalytic function. It has been shown that thiol group modification at CYS residues affects GST's enzymatic activity. Therefore, NO donor-mediated GST inactivation may possibly arise through interaction with either CYS or TYR residues, or both. It has also been reported that hydrogen peroxide leads to the formation of intra- or inter-subunit disulfide bonds between certain CYS residues within GST amino acid sequences, inactivating this enzyme [Letelier et al., 2006].

For the first time in the literature, our data suggest that NO compromises the detoxification abilities of cartilage and renders it more vulnerable to oxidant injuries through down-regulation of GST activity. GSTA4-4, an isoform of GST, is one of the most important pathways for HNE detoxification by catalyzing the conjugation of this aldehyde with GSH [Awasthi et al., 2004; Balogh and Atkins, 2011]. We have reported recently that gene silencing of GSTA4-4 by small interfering RNA augments the cytotoxic effect of HNE [Vaillancourt et al., 2008]. In contrast, the over-expression of this enzyme in chondrocytes offers significant protection against HNE-induced cell cytotoxicity. Our unpublished data show that GSTA4-4 over-expression in OA chondrocytes blocks different HNE-induced factors known to be involved in the OA process, including MMP-13 and COX-2 expression. Furthermore, we found, in the present study, that L-NIL prevents HNE-induced apoptosis as well as MMP-13 and PGE_2 production. The molecular mechanism underlying HNE-induced apoptosis as well as PGE_2 and MMP-13 was reported by us previously [Morquette et al., 2006; Vaillancourt et al., 2007; Vaillancourt et al., 2008]. These data can be explained by decreased HNE bioavailability via GST metabolism or perhaps via [HNE/L-NIL] adduct formation. As proposed in Figure 7, we can speculate that HNE trapping by L-NIL occurs via its LYS residue. The α,β double bond of HNE is believed to react spontaneously via Michael-type addition with the sulfhydryl group of CYS, the ε-amino group of LYS, and the imidazole function of HIS, and these adducts are currently considered as the predominant modification of proteins by HNE [Poli et al., 2008].

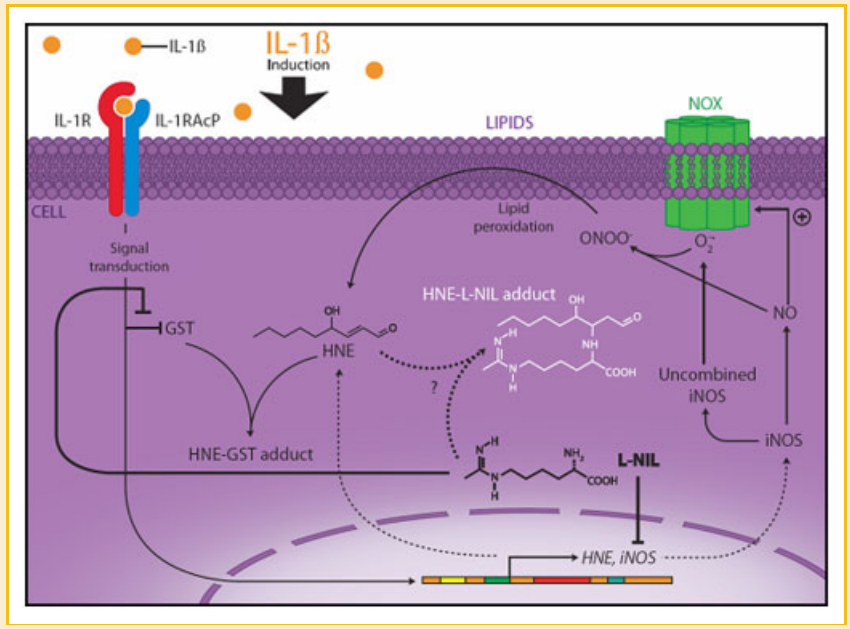

Fig. 7. Overview of different mechanisms mediating 4-hydroxynonenal (HNE) production and N-iminoethyl-L-lysine (L-NIL) action. Superoxide anion could be produced by NADPH oxidase (NOX), under NO effect, or by NOS uncoupling. NO mainly interacts with superoxide anion to form peroxynitrite, which further induces lipid peroxidation (LPO). The iNOS inhibitor L-NIL has the ability to abolish HNE levels via (i) NO reduction, (ii) possible [HNE/L-NIL] adduct formation, and (iii) GST up-regulation.

Furthermore, besides the upstream products of LPO, L-NIL abolished IL-1β-induced ROS generation and p47 NOX phosphorylation in chondrocytes. Two hypotheses could explain these data. The first is based on findings in the literature indicating that NO is important in ROS generation via NOX up-regulation. Compelling but controversial studies have investigated the role of NO in the regulation of ROS production via NOX. Our data are in agreement with the observations of Kaur et al. [2004] who reported NOX induction by NO in IL-1β-treated human coronary artery smooth muscle cells. Owayed et al. [2008] noted an increase in the activity and transcriptional regulation of NOX-1 and gp91phox by NO in peripheral blood lymphocytes from asthmatic patients. However, other studies presented additional evidence that prolonged exposure of human endothelial cells to NO donors causes sustained suppression of superoxide production via inhibition of NOX activity through S-nitrosylation of p47phox [Selemidis et al., 2007; Harrison et al., 2010]. Therefore, the second hypothesis is based on the ability of NOS to generate ROS (Fig. 7). The enzyme can produce large amounts of ROS when deprived of its critical co-factor BH_4 or its substrate L-arginine. Limitation of substrate availability leads to iNOS uncoupling and ultimately to ROS formation [Gielis et al., 2011]. Increased iNOS expression in the presence of limited substrate availability may result in iNOS uncoupling [Wells and Holian, 2007; Otani, 2009], thus potentially promoting ROS formation and, consequently, LPO. It has been reported that iNOS inhibition by N-(3-(aminomethyl)benzyl)acetamidine attenuates superoxide anion formation and oxidative damage [Heusch et al., 2010]. In this state, often referred to as NOS uncoupling, electron flow through the enzyme culminates in reduced molecular oxygen at the prosthetic heme site rather than NO formation [Abu-Soud et al., 1994].

In summary, our study demonstrated that L-NIL inhibited NO and ROS generation and, consequently, LPO in OA chondrocytes. Moreover, L-NIL administration was shown to play a role in redox status normalization via the prevention of GST inactivation in these cells. Our findings suggest that L-NIL may represent a new therapeutic approach to chronic autoimmune arthritis. An overview of the different mechanisms mediating HNE production and L-NIL action is proposed (Fig. 7).

REFERENCES

Abramson SB. 2008. Nitric oxide in inflammation and pain associated with osteoarthritis. Arthritis Res Ther 10(Suppl 2):S2.

Abu-Soud HM, Yoho LL, Stuehr DJ. 1994. Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and inter-domain electron transfer. J Biol Chem 269(51):32047–32050.

Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M. 1986. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 29(8):1039–1049.

Amin AR, Abramson SB. 1998. The role of nitric oxide in articular cartilage breakdown in osteoarthritis. Curr Opin Rheumatol 10(3):263–268.

Amin AR, Di Cesare PE, Vyas P, Attur M, Tzeng E, Billiar TR, Stuchin SA, Abramson SB. 1995. The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: Evidence for up-regulated neuronal nitric oxide synthase. J Exp Med 182(6):2097–2102.

Awasthi YC, Yang Y, Tiwari NK, Patrick B, Sharma A, Li J, Awasthi S. 2004. Regulation of 4-hydroxynonenal-mediated signaling by glutathione S-transferases. Free Radic Biol Med 37(5):607–619.

Balogh LM, Atkins WM. 2011. Interactions of glutathione transferases with 4-hydroxynonenal. Drug Metab Rev 43(2):165–178.

Carrico KM, Vaishnav RA, Hall ED. 2009. Temporal and spatial dynamics of peroxynitrite-induced oxidative damage after spinal cord contusion injury. J Neurotrauma 26(8):1369–1378.

McCartney-Francis NL, Song X, Mizel DE, Wahl SM. 2001. Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. J Immunol 166(4):2734–2740.

Castro RR, Cunha FQ, Silva FS Jr, Rocha FA. 2006. A quantitative approach to measure joint pain in experimental osteoarthritis–evidence of a role for nitric oxide. Osteoarthritis Cartilage 14(8):769–776.

Cesareo E, Parker LJ, Pedersen JZ, Nuccetelli M, Mazzetti AP, Pastore A, Federici G, Caccuri AM, Ricci G, Adams JJ, Parker MW, Lo BM. 2005. Nitrosylation of human glutathione transferase P1-1 with dinitrosyl diglutathionyl iron complex in vitro and in vivo. J Biol Chem 280(51):42172–42180.

Chen Q, Liu SQ, Du YM, Peng H, Sun LP. 2006. Carboxymethyl-chitosan protects rabbit chondrocytes from interleukin-1beta-induced apoptosis. Eur J Pharmacol 541(1–2):1–8.

El-Bikai R, Welman M, Margaron Y, Cote JF, Macqueen L, Buschmann MD, Fahmi H, Shi Q, Maghni K, Fernandes JC, Benderdour M. 2010. Perturbation of adhesion molecule-mediated chondrocyte-matrix interactions by 4-hydroxynonenal binding: Implication in osteoarthritis pathogenesis. Arthritis Res Ther 12(5):R201.

Esterbauer H, Schaur RJ, Zollner H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11(1):81–128.

Gielis JF, Lin JY, Wingler K, Van Schil PE, Schmidt HH, Moens AL. 2011. Pathogenetic role of eNOS uncoupling in cardiopulmonary disorders. Free Radic Biol Med 50(7):765–776.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 126(1):131–138.

Grigolo B, Roseti L, Fiorini M, Facchini A. 2003. Enhanced lipid peroxidation in synoviocytes from patients with osteoarthritis. J Rheumatol 30(2):345–347.

Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. 2002. S-nitrosylation of matrix metalloproteinases: Signaling pathway to neuronal cell death. Science 297(5584):1186–1190.

Gu Z, Nakamura T, Lipton SA. 2010. Redox reactions induced by nitrosative stress mediate protein misfolding and mitochondrial dysfunction in neurodegenerative diseases. Mol Neurobiol 41(2–3):55–72.

Harrison CB, Drummond GR, Sobey CG, Selemidis S. 2010. Evidence that nitric oxide inhibits vascular inflammation and superoxide production via a p47phox-dependent mechanism in mice. Clin Exp Pharmacol Physiol 37(4):429–434.

Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M. 1998. Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthritis Rheum 41(7):1266–1274.

Heusch P, Aker S, Boengler K, Deindl E, van de SA, Klein K, Rassaf T, Konietzka I, Sewell A, Menazza S, Canton M, Heusch G, Di LF, Schulz R. 2010. Increased inducible nitric oxide synthase and arginase II expression in heart failure: No net nitrite/nitrate production and protein S-nitrosylation. Am J Physiol Heart Circ Physiol 299(2):H446–H453.

Kaur J, Dhaunsi GS, Turner RB. 2004. Interleukin-1 and nitric oxide increase NADPH oxidase activity in human coronary artery smooth muscle cells. Med Princ Pract 13(1):26–29.

Letelier ME, Martinez M, Gonzalez-Lira V, Faundez M, Aracena-Parks P. 2006. Inhibition of cytosolic glutathione S-transferase activity from rat liver by copper. Chem Biol Interact 164(1–2):39–48.

Letelier ME, Molina-Berrios A, Cortes-Troncoso J, Jara-Sandoval JA, Muller A, Aracena-Parks P. 2010. Comparative effects of superoxide anion and hydrogen peroxide on microsomal and cytosolic glutathione S-transferase activities of rat liver. Biol Trace Elem Res 134(2):203–211.

McInnes IB, Leung BP, Field M, Wei XQ, Huang FP, Sturrock RD, Kinninmonth A, Weidner J, Mumford R, Liew FY. 1996. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. J Exp Med 184(4):1519–1524.

Montezano AC, Touyz RM. 2012. Reactive oxygen species and endothelial function - role of nitric oxide synthase uncoupling and nox family nicotinamide adenine dinucleotide phosphate oxidases. Basic Clin Pharmacol Toxicol 110(1):87–94.

Morquette B, Shi Q, Lavigne P, Ranger P, Fernandes JC, Benderdour M. 2006. Production of lipid peroxidation products in osteoarthritic tissues: New evidence linking 4-hydroxynonenal to cartilage degradation. Arthritis Rheum 54(1):271–281.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63.

Nakagawa S, Arai Y, Mazda O, Kishida T, Takahashi KA, Sakao K, Saito M, Honjo K, Imanishi J, Kubo T. 2010. N-acetylcysteine prevents nitric oxide-induced chondrocyte apoptosis and cartilage degeneration in an experimental model of osteoarthritis. J Orthop Res 28(2):156–163.

Ng L, Nichols K, O'Rourke K, Maslen A, Kirby GM. 2007. Repression of human GSTA1 by interleukin-1beta is mediated by variant hepatic nuclear factor-1C. Mol Pharmacol 71(1):201–208.

Otani H. 2009. The role of nitric oxide in myocardial repair and remodeling. Antioxid Redox Signal 11(8):1913–1928.

Owayed A, Dhaunsi GS, Al-Mukhaizeem F. 2008. Nitric oxide-mediated activation of NADPH oxidase by salbutamol during acute asthma in children. Cell Biochem Funct 26(5):603–608.

Pelletier JP, Fernandes JC, Jovanovic DV, Reboul P, Martel-Pelletier J. 2001. Chondrocyte death in experimental osteoarthritis is mediated by MEK 1/2 and p38 pathways: Role of cyclooxygenase-2 and inducible nitric oxide synthase. J Rheumatol 28(11):2509–2519.

Pelletier JP, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, Di Battista JA, Martel-Pelletier J. 1998. Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. Arthritis Rheum 41(7):1275–1286.

Pelletier JP, Lascau-Coman V, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, Martel-Pelletier J. 1999. Selective inhibition of inducible nitric oxide synthase in experimental osteoarthritis is associated with reduction in tissue levels of catabolic factors. J Rheumatol 26(9):2002–2014.

Poli G, Schaur RJ, Siems WG, Leonarduzzi G. 2008. 4-hydroxynonenal: A membrane lipid oxidation product of medicinal interest. Med Res Rev 28(4):569–631.

Rosa SC, Judas F, Lopes MC, Mendes AF. 2008. Nitric oxide synthase isoforms and NF-kappaB activity in normal and osteoarthritic human chondrocytes: Regulation by inducible nitric oxide. Nitric Oxide 19(3): 276–283.

Selemidis S, Dusting GJ, Peshavariya H, Kemp-Harper BK, Drummond GR. 2007. Nitric oxide suppresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. Cardiovasc Res 75(2):349–358.

Shah R, Raska K Jr, Tiku ML. 2005. The presence of molecular markers of in vivo lipid peroxidation in osteoarthritic cartilage: A pathogenic role in osteoarthritis. Arthritis Rheum 52(9):2799–2807.

Sharma R, Ellis B, Sharma A. 2011. Role of alpha class glutathione transferases (GSTs) in chemoprevention: GSTA1 and A4 overexpressing human leukemia (HL60) cells resist sulforaphane and curcumin induced toxicity. Phytother Res 25(4):563–568.

Sidorkina O, Espey MG, Miranda KM, Wink DA, Laval J. 2003. Inhibition of poly(ADP-RIBOSE) polymerase (PARP) by nitric oxide and reactive nitrogen oxide species. Free Radic Biol Med 35(11):1431–1438.

Sosroseno W, Bird PS, Seymour GJ. 2011. Nitric oxide production by a murine macrophage cell line (RAW264.7 cells) stimulated with Aggregatibacter actinomycetemcomitans surface-associated material. Anaerobe 17(5): 246–251.

Squadrito GL, Pryor WA. 1998. Oxidative chemistry of nitric oxide: The roles of superoxide, peroxynitrite, and carbon dioxide. Free Radic Biol Med 25(4–5):392–403.

Szabo C, Modis K. 2010. Pathophysiological roles of peroxynitrite in circulatory shock. Shock 34(Suppl 1):4–14.

Uchida K. 2003. 4-Hydroxy-2-nonenal: A product and mediator of oxidative stress. Prog Lipid Res 42(4):318–343.

Vaillancourt F, Fahmi H, Shi Q, Lavigne P, Ranger P, Fernandes JC, Benderdour M. 2008. 4-Hydroxynonenal induces apoptosis in human osteoarthritic chondrocytes: The protective role of glutathione-S-transferase. Arthritis Res Ther 10(5):R107.

Vaillancourt F, Morquette B, Shi Q, Fahmi H, Lavigne P, Di Battista JA, Fernandes JC, Benderdour M. 2007. Differential regulation of cyclooxygenase-2 and inducible nitric oxide synthase by 4-hydroxynonenal in human osteoarthritic chondrocytes through ATF-2/CREB-1 transactivation and concomitant inhibition of NF-kappaB signaling cascade. J Cell Biochem 100(5):1217–1231.

Veihelmann A, Landes J, Hofbauer A, Dorger M, Refior HJ, Messmer K, Krombach F. 2001. Exacerbation of antigen-induced arthritis in inducible nitric oxide synthase-deficient mice. Arthritis Rheum 44(6):1420–1427.

Wakita C, Honda K, Shibata T, Akagawa M, Uchida K. 2011. A method for detection of 4-hydroxy-2-nonenal adducts in proteins. Free Radic Biol Med 51(1):1–4.

Wells SM, Holian A. 2007. Asymmetric dimethylarginine induces oxidative and nitrosative stress in murine lung epithelial cells. Am J Respir Cell Mol Biol 36(5):520–528.

Xia W, Szomor Z, Wang Y, Murrell GA. 2006. Nitric oxide enhances collagen synthesis in cultured human tendon cells. J Orthop Res 24(2):159–172.

Zhou R, Yazdi AS, Menu P, Tschopp J. 2011. A role for mitochondria in NLRP3 inflammasome activation. Nature 469(7329):221–225.