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In vitro study of the antioxidant properties of nimesulide and 4-OH nimesulide: effects on HRP- and luminol-dependent chemiluminescence produced by human chondrocytes

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Summary

Objectives: Reactive oxygen species (ROS) are now recognized to play an important role in the pathogenesis of rheumatic diseases and constitute an interesting therapeutic target for drugs. This *in vitro* study was designed to evaluate the antioxidant properties of nimesulide (NIM), a nonsteroidal antiinflammatory drug of the sulfonanilide class, and its main metabolite 4-OH nimesulide (4-OHNIM).

Methods: The scavenging effects of NIM and 4-OH NIM on hydroxyl radical ($^{\cdot}OH$) and superoxide anions ($O_2^{\cdot-}$) were investigated by electron spin resonance (ESR), using 5,5-dimethylpyrroline-N-oxide (DMPO) as the spin trap agent. The quenching properties of these drugs on hypochlorite anion was studied by luminol enhanced chemiluminescence. Finally, the effects of NIM and 4-OHNIM on the reactive oxygen species production by human articular chondrocytes were recorded by HRP and luminol-enhanced chemiluminescence.

Results: By this method it has been demonstrated that NIM and 4-OHNIM, at concentrations ranging from 10 to 100 μM, are potent scavengers of $^{\cdot}OH$ whereas only 4-OHNIM was capable to scavenge $O_2^{\cdot-}$. Chemiluminescence generated by HOCl was also significantly and dose-dependently inhibited by both NIM and 4-OHNIM. Nevertheless, at each concentration tested, the inhibitory effect of 4-OHNIM was significantly more marked, even at the highest concentration (100 μM). Furthermore, when chondrocytes were pre-incubated for 48–96 h with NIM or 4-OHNIM, the luminol- and HRP-dependent CL produced by the cells was significantly inhibited in a dose-dependent manner.

Conclusions: NIM and 4-OHNIM may protect cartilage against oxidative stress, not only by scavenging ROS but also by inhibiting their production by chondrocytes. © 2000 OsteoArthritis Research Society International

Key words: Antioxidant, Chondrocytes, Nimesulide, Chemiluminescence.

Introduction

Nimesulide (NIM) is a non-steroidal antiinflammatory drug (NSAID) commonly used in the treatment of osteoarthritis (OA).[1,2] The compound is weakly acidic (pka=6.5) and differs from other NSAIDs in that its chemical structure contains a sulfonanilide moiety as the acidic group.[3] In humans, the drug is mostly biotransformed into 4-hydroxynimesulide (4-OHNIM), a metabolite which appears to contribute to the antiinflammatory activity of the compound.[4] NIM appears to exert its antiinflammatory activity through a variety of mechanisms, including a relatively selective inhibition of prostaglandin synthesis by COX-2,[5–11] the inhibition of histamine release from human basophils and tissue mast cells,[12] the reduction of neutrophil aggregation[13] and adherence to endothelial cells, as well as the inhibition of PAF (platelet activating factor) production by stimulated human neutrophils.[14] Moreover, NIM reduces the generation of superoxide anion by stimulated polymorphonuclear leukocytes through the inhibition of protein kinase C translocation and phosphodiesterase type IV activity, the principal enzyme responsible for the degradation of leucocyte cAMP.[15] In a study assessing the antioxidant activity of NIM and its main metabolites, it was reported that 4-OHNIM possessed antilipoperoxidant activity at a much lower concentration than the parent compound and another metabolite recently identified in human urine, 2-(4′-hydroxyphenoxy)-4-N-acetylaminomethansulfonanilide.[16] Using an *in vitro* model of erythrocyte membrane lipoperoxidation induced by cumene hydroperoxide (CuOOH), it was demonstrated that 4-OHNIM protects erythrocyte membrane by directly quenching peroxyl and alkoxyl radical species.[17] NIM is also a potent scavenger of hypochlorous acid which is responsible for large part of tissue injury and inflammation mediated by PMNs.[18] Nimesulide also exhibited favorable hydroxyl radical scavenging activity in a membrane phosphatidylcholine liposome.[19] Recently, NIM was reported to induce the intracellular phosphorylation and activation of glucocorticoid receptors and to activate their binding to target genes in human synovial fibroblasts.[20] This mechanism may contribute largely to its antiinflammatory activity.

In addition to its antiinflammatory action, nimesulide may also exert beneficial effects on osteoarthritic cartilage metabolism. When added to human articular cartilage explant *in vitro*, NIM at a therapeutic concentration (10 μM), reduced the degradation of the matrix by inhibiting the synthesis of metalloproteinases such as collagenase and stromelysin.[21] Using primary human chondrocytes cultured in suspension, we showed that therapeutic concentrations

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of NIM could inhibit the synthesis of IL-6, while it did not modify proteoglycan or IL-8 production.[22] With human osteoarthritic synovial fibroblasts in culture, NIM also reduced the synthesis of urokinase and increased plasminogen activator inhibitor-1 (PAI-1), suggesting that this drug decreases the degradation of cartilage matrix not only by reducing metalloproteases synthesis but also by inhibiting one of the mechanisms involved in metalloprotease activation.[23]

The antioxidant properties of NIM suggest that this drug could also protect cartilage by inhibiting reactive oxygen species (ROS) production by chondrocytes. Recently, it was reported that chondrocytes possess an NADPH oxidase complex[24,25] and produce ROS in response to phorbol 12-myristate 13-acetate (PMA), calcium ionophore ionomycin, anoxia-reoxygenation cycles and cytokines (TNFα, IL-1β, IFNγ).[26–29] ROS are able to degrade matrix components (e.g. type II collagen, proteoglycans, hyaluronic acid) and DNA by direct action[30–32] and by indirect activation of latent collagenase.[33] Furthermore, α1-proteinase inhibitor, the major inhibitor of elastase, is inactivated by exposure to the PMNs-derived oxygen radicals species,[34] awakening defences against the degradative actions of proteases on connective tissues. Exposure of the chondrocytes to oxygen radicals inhibits protein and DNA synthesis in these cells,[35] and depletes intracellular ATP as a result of a simultaneous inactivation of glyceraldehyde-3-phosphate dehydrogenase.[36] These findings suggest that ROS produced by chondrocytes could play a key role in cartilage degradation and, therefore, constitute a potential target for drugs, such as NSAIDs, that are used in the treatment of joint diseases and possess antioxidant side-effects.

In this work, we have investigated the capacities of NIM and 4-OHNIM to scavenge hypochlorous anion, hydroxyl radicals and superoxide anion and to modulate the production of ROS by human articular chondrocytes.

Materials and methods

HUMAN CHONDROCYTES IN CULTURE

Cartilage was obtained from the knees of eight adult donors shortly after death (three males and five females with a mean age of 58 years), being excised from the superficial and medium layers and avoiding the calcified layer. Chondrocytes were cultured for a short period in order to maintain their phenotype. Cartilage was cut into small fragments and then subjected to enzymatic digestion sequentially with hyaluronidase, pronase and collagenase. First, the fragments were incubated for 30 min at 37°C under constant agitation (200 rpm) with hyaluronidase (Sigma Chemie, Bornem, Belgium) dissolved (0.5 mg/ml) in Dulbecco's modified Eagle's medium (DMEM, Biowhittaker, Bruxelles, Belgium) (10 ml enzyme solution per 3 g of cartilage). The fragments were then incubated for 1 h at 37°C in pronase solution (1 mg/ml of DMEM; enzyme obtained from Merck-Belgolabo, Overijse, Belgium) (3 g cartilage per 10 ml enzyme solution). Finally, the cartilage fragments were incubated for 20 h at 37°C under constant agitation (200 rpm) with collagenase (Sigma Chemie) dissolved in DMEM (1 mg/ml) containing 1% of Ultroser G (Gibco, Gent, Belgium) (3 g cartilage per 10 ml enzyme solution). Ultroser G is a serum substitute designed to replace fetal calf serum in nutrient culture medium. The cells were then filtered through nylon mesh with a pore diameter of 70 μm, washed three times, counted (5×10^6 cells/ml) and resuspended in 10 ml of DMEM supplemented with 1% of ITS$^+$. ITS$^+$ is a well defined culture supplement containing 0.625 mg/ml insulin, 0.625 mg/ml transferrin, 0.625 μg/ml selenium acid, 125 mg/ml bovine serum albumin (BSA) and 0.535 mg/ml linoleic acid. Cells were then kept at 37°C for 48 h under constant agitation.

After this washout period, cells (0.5×10^6 cells/ml) were then seeded in 24-well plates, treated for low cell attachment and cultured for 24, 48, 72 or 96 h in DMEM without red phenol containing 1% ITS$^+$, 1 mM glutamine and the drugs tested. NIM and 4-OHNIM (Therabel Research, Bruxelles, Belgium) were added to the culture medium at concentrations ranging from 0.3 to 30 μg/ml. The peak concentrations of nimesulide reported in the plasma of RA patients who were taking 100 mg orally twice a day ranged from 10 to 20 μM. After a single or repeated 100 mg doses b.i.d., 10 μM was above the highest level noted in the synovial fluid of patients receiving 100 mg b.i.d. orally for 7 days, 3 h after the last dose.[37] On the other hand, the mean plasma concentration of 4-OHNIM after single-dose administration of NIM 100 mg tablets to 12 healthy male volunteers was 1.4 mg/l.[38] NIM and 4-OHNIM were dissolved in 0.1 M NaOH (10 mg/ml) and diluted in DMEM (without red phenol) at the concentration of 10 μg/ml. pH was adjusted slowly back to 7.4 with 0.1 M HCl. After incubation, the chondrocytes suspension was collected by centrifugation and washed three times with HBSS (pH 7.4). The chondrocyte pellets were resuspended in 4.0 ml of HBSS (pH 7.4) and mixed gently (cells density $\pm5\times10^4$/500 μl). A 500 μl chondrocyte suspension was added to a polypropylene cuvette for CL measurement.

ELECTRON SPIN RESONANCE (ESR) EXPERIMENTS

The potential scavenging effect of NIM and 4-OHNIM on $O_2^{\cdot-}$ and $^{\cdot}OH$ was investigated by direct addition of NIM and 4-OHNIM to the $O_2^{\cdot-}$ or $^{\cdot}OH$ generating systems. Hydroxyl radicals ($OH^{\cdot}$) were generated by the Fenton reagents resulting from the mixture of the following agents: 0.1 mM H_2O_2, 0.01 mM $FeSO_4.7H_2O$ and 10 μM EDTA (Merck-Belgolabo, Overijse, Belgium). On the other hand, superoxide anion was produced from xanthine (1 mM)-xanthine-oxidase (0.08 U/ml) reaction in 50 mM phosphate buffer (pH 7.8). The scavenging effect of NIM and 4 OHNIM on $^{\cdot}OH$ and $O_2^{\cdot-}$ was monitored by EPR trapping technique, using 5,5-dimethylpyrroline-N-oxide (DMPO; 100 mM; Sigma-Aldrich, Bornem, Belgium) as the spin trap agent. After preparation, the mixture was immediately transferred to a flat cell and placed in the TM 110 cavity of the ESR spectrometer (final volume 1 ml). The spectra were recorded at room temperature using a Bruker ESP 300 E spectrometer (Karlsruhe, Germany) operating at X-band with 100 kHz modulation frequency. These measurements were performed with non-saturating 20 mW microwave power and microwave frequency 9.75 GHz. The instrumental settings were: center field 3480 G, time constant 40.96 ms, time conversion 40.96 ms, receiver gain 2×10^4 and sweep width 100 G.

CHEMILUMINESCENCE (CL) MEASUREMENT

The *in vitro* effects of NIM and 4-OHNIM on the light emission induced by sodium hypochlorite solution (NaOCl; 10^{-4} M; BDH Laboratory, London, U.K.) were investigated.

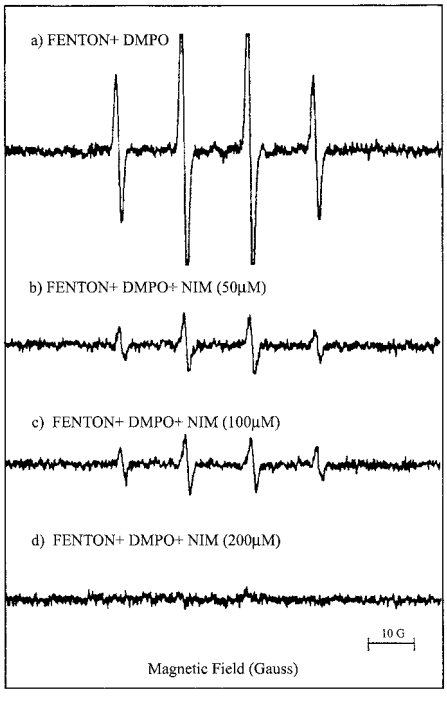

Fig. 1. ESR spectra (DMPO 100 mM) of the spin adduct of hydroxyl radical generated by the Fenton reaction (H_2O_2 0.1 mM, $FeSO_4.7H_2O$ 0.01 mM, EDTA 0.01 mM). (a) Normal spectrum (hyperfine splittings for DMPO-˙OH adduct: aH=aN=14.87 G); (b) DMPO-˙OH spectrum in the presence of NIM 50 μM; (c) DMPO-˙OH spectrum in the presence of 100 μM; (d) DMPO-˙OH signals was fully inhibited by NIM 200 μM.

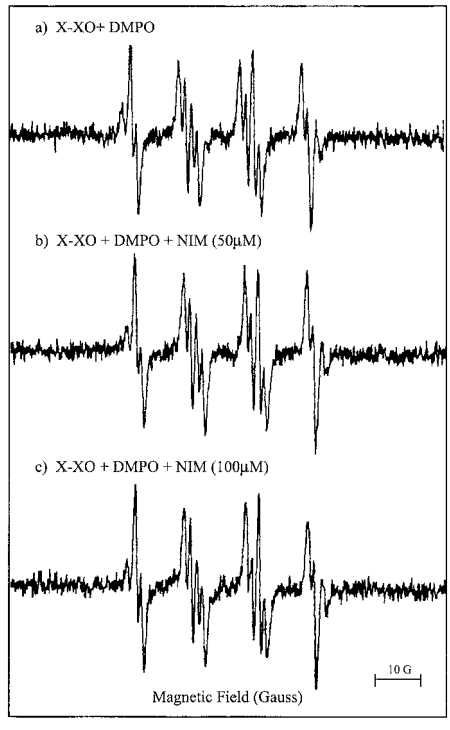

Fig. 2. ESR spectra (DMPO 100 mM) of superoxide adduct (DMPO-OOH) generated by xanthine (1 mM)/xanthine oxidase (0.08 mU/ml) system in 50 mM phosphate buffer pH 7.4. (b,c) Complete system xanthine/xanthine-oxidase generating superoxide radical in the presence of NIM 50 μM (b) or 100 μM (c). No significant variation of the ESR signals was observed in the presence of NIM.

The assays were performed at 25°C in phosphate buffer (pH 7.4). Light emission was produced by the direct reaction of NaOCl (10^{-4} M) with luminol (2.5×10^{-5} M; 5-amino-2, 3-dihydro-1, 4-phthalazinedione). Chemiluminescence generated by chondrocytes was also recorded according the following protocol: luminol (5 μM), chondrocyte suspension (5.10^4 cells/0.5 ml) and horseradish peroxidase (HRP, 0.05 U/0.5 ml) were sequentially added to a circular polystyrene reaction vessel. To verify that light emission was the consequence of reactive oxygen species production, superoxide dismutase (SOD; 0.01–100 U/0.5 ml) and catalase (0–500 U/0.5 ml) were added to the reaction mixture. CL followed at 37°C for 10 min. CL was measured with a BioOrbit 1251 Luminometer (Turku-Finland) and the data were expressed in arbitrary units (1U=1 mV).

STATISTICAL ANALYSIS

The mean±S.D. for each variable was calculated. Comparison of the treated groups with the controls was performed using the unpaired Student's *t*-test, with a significant limit set at $P<0.05$.

Results

SCAVENGING EFFECTS OF NIM AND 4-OHNIM ON HO AND $O_2^{\cdot-}$ RADICALS

The reaction of DMPO with ˙OH generated by the Fenton reaction ($FeSO_4$ and H_2O_2) resulted in a four-line ESR spectrum (DMPO-˙OH adduct). This ESR signal decreased in the presence of increasing concentrations (10–200 μM) of NIM (IC_{50}=5.2 μM) and 4-OHNIM (IC_{50}=4.9 μM) (Fig. 1). In contrast, no inhibiting effect was observed on the $O_2^{\cdot-}$ produced by the xanthine/xanthine oxidase system in the presence of NIM (Fig. 2) while, at similar doses, its metabolite (4-OHNIM) caused a decrease of the ESR spectrum intensity (IC_{50}=17 μM) [Fig. 3(a), (b)]. At 10 μM, which corresponds at the peak plasmatic concentration after oral administration of a therapeutic dose of NIM, ESR signal height generated by ˙OH was reduced by 70%, whereas $O_2^{\cdot-}$ ESR signal was unaffected. Inversely, at 10 μM, 4-OHNIM decreased $O_2^{\cdot-}$ ESR signal height by 38%.

SCAVENGING EFFECTS OF NIM AND 4-OHNIM ON CHEMICALLY-INDUCED CHEMILUMINESCENCE

The chemiluminescence generated by OHCl was dose-dependently inhibited by NIM (IC_{50}>100 μM) and 4-OHNIM (IC_{50}=17 μM). Nevertheless, at each concentration tested the inhibition was significantly higher in the presence of 4-OHNIM than NIM ($0.01<P<0.001$) (Fig. 4). At therapeutic concentration (25 μM), NIM slighly quenched (−16%) OHCl whereas 4-OHNIM decreased light emission by 70%. At 100 μM, the inhibition of OHCl-induced CL is almost complete for 4-OHNIM (−95%) but not for NIM (only 38% of inhibition).

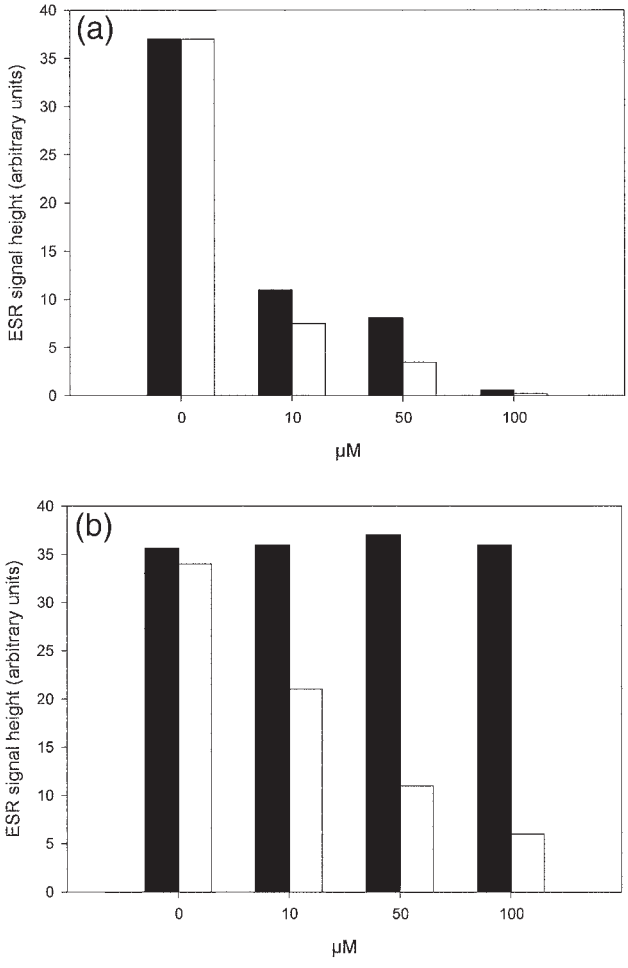

Fig. 3. Effects of NIM (black columns) and 4-OHNIM (white column) on ESR signal heights (DMPO 100 mM) generated by hydroxyl radicals resulting from the Fenton reaction (a) and by superoxide radical produced by xanthine/xanthine-oxidase-system (b).

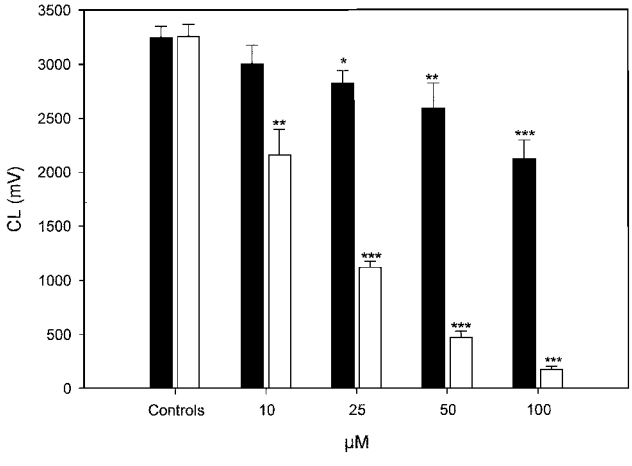

Fig. 4. Effects of increase concentrations of NIM (black column) and 4-OHNIM (white column) on the peak value of luminol (10^{-4} M)-enhanced chemiluminescence curve generated by OHCl (NaOCl). Results are expressed as the mean values of triplicate. Statistically significant difference compared with the controls: *P<0.05; **P<0.01; ***P<0.001.

EFFECTS OF NIM AND 4-OHNIM ON LUMINOL AND HRP-DEPENDENT CHEMILUMINESCENCE

To verify that the chemiluminescence resulted from the production of ROS by chondrocytes, we introduced two antioxidant enzymes, superoxide dismutase (SOD) and catalase, into the reaction mixture (Fig. 5). Interestingly, CL generated by chondrocytes was dose-dependently depressed by both, catalase (R=0.96; P=0.01; y=−23.02x+51.8) and superoxide dismutase (R=0.91; P=0.0125; y=−12.8x+22.6).

With the aim of evaluating the effects of NIM and its metabolite, 4-OHNIM, on the ROS generating systems of living cells, chondrocytes were pre-incubated for 24–96 h with increased amounts of NIM and 4-OHNIM. At concentrations ranging from 0.4 to 10 µg/ml, NIM inhibited in a time- and dose-dependent manner CL generated by chondrocytes (Fig. 6). In the therapeutic range of concentrations (6–12 µM), the inhibition was already superior at 50%. After 48 h of incubation, 4-OHNIM also decreased chemiluminescence but to a lesser extent than NIM (Fig. 7). At plasmatic concentrations of 4-OHNIM the inhibition of the HRP- and luminol-enhanced chemiluminescence produced by chondrocytes was less than 50% even after 96 h of incubation.

Discussion

Reactive oxygen species have been shown to play a significant role in the degradation of cartilage in arthritis. Recent studies suggested that rabbit, bovine, or porcine chondrocytes are potent sources of oxygen free radicals.[25,27,29] Previous work from our laboratory showed that isolated human chondrocytes were also capable of producing reactive oxygen species, such as hydrogen peroxide and hydroxyl radicals, when they were submitted to anoxia-reoxygenation cycle or PMA stimulation.[28]

As demonstrated by ESR method, NIM is a potent scavenger of hydroxyl radical, but is ineffective on superoxide anions. In contrast, 4-OHNIM quenched both $^{\cdot}OH$ and $O_2^{\cdot-}$. This finding can be explained by the presence of a phenolic structure in 4-OHNIM which is particularly susceptible to further $O_2^{\cdot-}$ attack. In a previous study, Maffei-Facino *et al*. demonstrated that NIM and its metabolites could prevent $^{\cdot}OH$-induced depolymerization of hyaluronic acid and that the phenolic metabolite displayed maximun scavenging activity in the peroxidation model, where the proximate reactive damaging species were oxygen- ($RO^{\cdot}$ and $ROO^{\cdot}$) and carbon-centered ($R^{\cdot}$) lipid radicals.[16] Furthermore, since we know that NIM and 4-OHNIM can bind Fe^{2+}, the mechanism of the action of NIM on the Fenton reaction could be the chelation of Fe^{2+}.[16,17] This chelating property of NIM and 4-OHNIM may also contribute to its therapeutic potency by reducing 'free' iron at the inflammatory site when inflammatory stimuli induce respiratory bursts of invading leukocytes.[39,40,41]

Neutrophils, recruited to tissue sites of inflammation, release a variety of oxidants and enzymes, that are responsible for tissue damage. Among the oxidants released are potent chlorinated compounds, such as hypochlorous acid, which induce tissue cell damage and inactivate protease inhibitors, particularly α1-antiprotease, the specific inhibitor of neutrophil elastase. In our study, NIM and 4-OHNIM quenched hypochlorous acid in a dose-dependent manner,

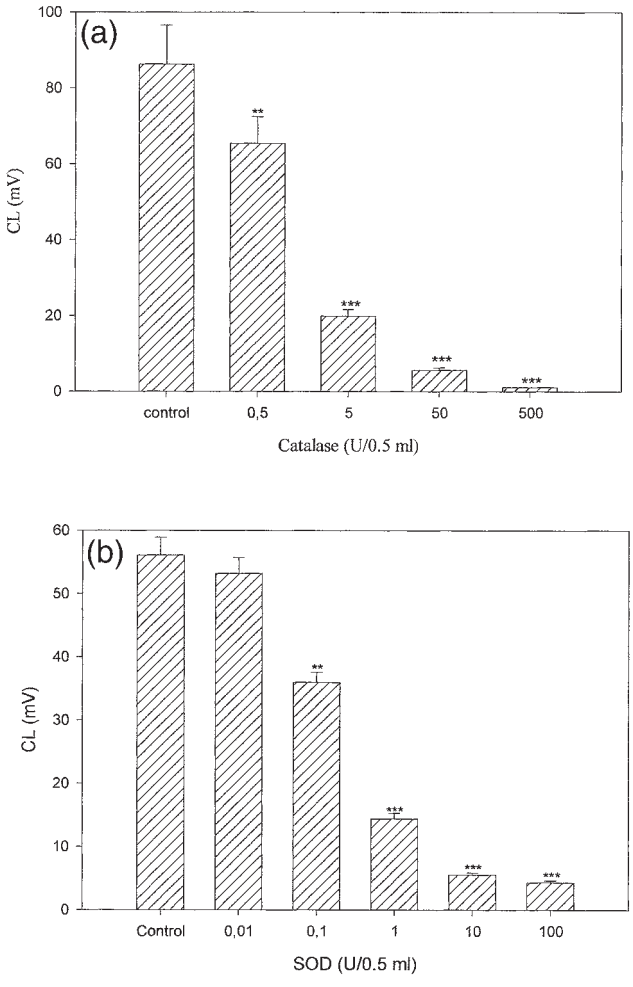

Fig. 5. Effects of increase amounts of catalase (a) and superoxide dismutase (b) on HRP- and luminol-enhanced chemiluminescence. Luminol was used at the concentration of 5 μM and the reaction was started by the injection of 0.05 U of HRP.

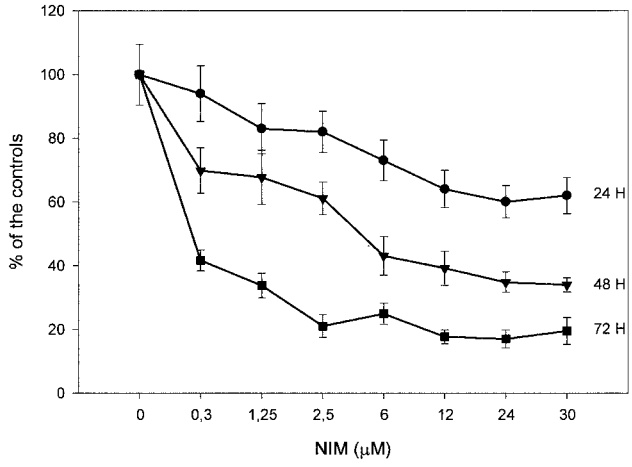

Fig. 6. Effects of increasing concentrations of NIM on HRP- and luminol-enhanced chemiluminescence generated by human chondrocytes. Chondrocytes were pre-incubated for 24 (—●—), 48 (—▼—) or 72 (—■—) h with nimesulide. Results are means of five experiments conducted with chondrocytes isolated from cartilage of five different donors expressed as the percentage of the control values. Statistically significant difference compared with the controls: *P<0.05; **P<0.01; ***P<0.001.

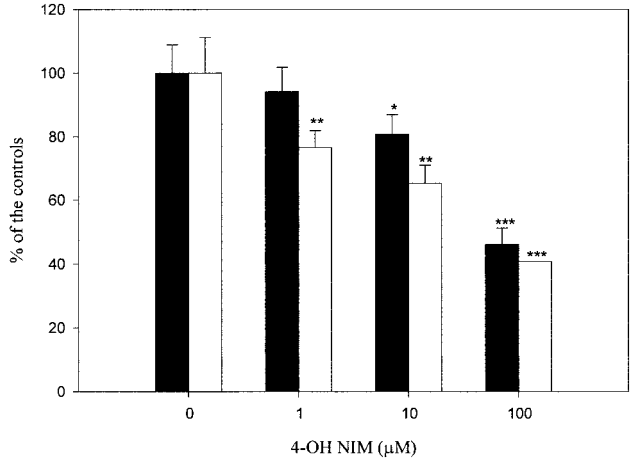

Fig. 7. Effects of increase concentrations of 4-OHNIM on HRP- and luminol-enhanced chemiluminescence generated by human chondrocytes. Chondrocytes were pre-incubated for 48 h (black columns) or 96 h (white columns) with nimesulide metabolite. Results are means of three experiments conducted with chondrocytes isolated from cartilage of three different donors. For each individual experiment, four culture flasks were used for each concentration of 4-OHNIM as well as for the corresponding controls. Results are expressed as the percentage of the control values. Statistically significant difference compared with the controls: *P<0.05; **P<0.01; ***P<0.001.

as demonstrated by the luminol-enhanced chemiluminescence method. Nevertheless, at each concentration tested the inhibition was significantly more marked in the presence of 4-OHNIM than in that of NIM. Other studies have also shown that NIM, at therapeutic concentrations (10–100 μmol/l), prevented the inactivation of α1-antiprotease by neutrophils by effectively scavenging the hypochlorous acid released by neutrophils.[42] In contrast, aspirin and naproxen were completely ineffective, suggesting that the presence of an NH group, particularly exposed at the surface of the NIM and therefore prone to chlorination by OHCl, probably accounts for the observed effect of NIM.[42] On the other hand, our results also demonstrate that 4-OHNIM is a stronger OHCl scavenger than NIM, suggesting that the OH group could also react with OHCl. At 10 μmol/l (equal to 3.08 mg/l) which corresponds to the level of NIM found in plasma after a single 100 mg oral dose, NIM and 4-OHNIM inhibited OHCl-induced chemiluminescence by 11% and 38% respectively, suggesting that *in vivo*, 4-OHNIM could be the active compound in this process.

The production of ROS by isolated chondrocytes can be estimated by measurement of the chemiluminescence.[27,29] Due to the low quantum yield of ROS, it is difficult to measure the intrinsic CL produced by chondrocytes. Luminescence enhancers such as luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) and lucigenin are susceptible to oxygenations and of high CL yield. Luminol-dependent or lucigenin-dependent CL in articular chondrocytes has been established by Rathakrishnan *et al*.[27,29] Luminol-dependent CL is considered to reflect the production of hydrogen peroxide, peroxynitrite, superoxide and singlet oxygen.[43–47] In this study, we have developed an HRP- and luminol-dependent CL assay to measure the production of ROS by cultured human

chondrocytes. Both luminol and HRP are necessary to the CL measurement. In the absence of these chemiluminescence enhancers, chondrocytes failed to emit chemiluminescence. Both catalase and superoxide dismutase fully suppressed chemiluminescence generated by chondrocytes, indicating that this assay measures the production of hydrogen peroxide and superoxide anions by chondrocytes. When chondrocytes were pre-incubated with NIM and 4-OHNIM, HRP- and luminol-dependent chemiluminescence was dose- and time-dependently decreased. These findings suggest that NIM not only acts as a scavenger of ROS, but also inhibits the intracellular generating system of ROS. At the present time, two mechanisms have been advanced to explain the inhibitory effect of NIM on ROS production by leukocytes. By analogy, we can speculate that the effect of NIM on oxygen free radicals (superoxide anion, $O_2^{\cdot-}$) may be related to its inhibitory activity on cytosolic phosphodiesterase type IV (cAMP-specific), thus increasing cytosolic cAMP and, as a consequence, protein kinase A activity, which promotes the phosphorylation of a number of intracellular factors and inhibits the assembly of NADPH-oxidase in the plasma membrane.[15] Another mechanism of the action of NIM could be direct interference with the adenosine receptor system on the cell membrane. Recently, it was demonstrated that the pre-incubation of cells with the specific adenosine receptor antagonist theophylline was able to significantly reverse the inhibitory activity of NIM on free radicals production sustained by FMLP and the ionophores,[45] suggesting the possibility that NIM interferes directly with the adenosine receptor system on the cell membrane.

In conclusion, our study demonstrates for the first time that an NSAID may inhibit ROS production by human chondrocytes. By this way, nimesulide could locally protect cartilage against the deleterious effects of ROS on the cartilage matrix integrity. This study also shows that the parent drug nimesulide, and its metabolite, may have different scavenging effects, suggesting that drug metabolization could also influence the drug efficacy. Finally, we must keep in mind that the reaction of NSAIDs with ROS could also conduct to the formation of transient radical species which could act as electron donors and initiate oxidative chain reaction.[48] The presence of excited state of the NSAID should be researched before a definitive conclusion is reached on their antioxidant properties.

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