Reaction of Hypochlorous Acid with Bovine Nasal Cartilage Comparison to Pig Articular Cartilage

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Dedicated to Professor Dr. Peter Welzel on the occasion of his 60th birthday

Hypochlorous Acid, Bovine Nasal Cartilage, Proton-NMR Spectroscopy, Cartilage Degradation

The action of sodium hypochlorite (NaOCl) on bovine nasal cartilage was studied by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy in order to model degradation processes of cartilage caused by neutrophil-derived hypochlorous acid.

Nasal cartilage was chosen as a mean of comparison because it differs from articular cartilage in its composition. It contains some more proteoglycans, i.e. polymeric carbohydrates and less collagen than articular cartilage. This is important for studying the influence of hypochlorous acid on cartilage components (collagen and polysaccharides). Cartilage samples were incubated at 37 °C with phosphate buffer in the presence or absence of NaOCl. Supernatants were collected and assayed by NMR-spectroscopy. In the presence of pure phosphate buffer, the supernatants of bovine nasal cartilage were less rich in low molecular mass metabolites (e.g. amino acids, lactate) than articular cartilage. However, intense signals for highly mobile N acetyl groups of cartilage polysaccharides were detectable in nasal cartilage.

NaOCl caused an increase in signals for acetate and formiate. Signals for N-acetyl groups rose only during the first 25 minutes of incubation with NaOCl. Then, their concentration decreased markedly. These changes were related to an enhanced release of chondroitinsulfate from nasal cartilage.

Introduction

A large amount of polymorphonuclear leukocytes (PMNs) accumulates in pathologically changed synovial fluids of patients suffering from rheumatic diseases, especially rheumatoid arthritis (Brown, 1988). It is assumed that PMNs are involved in the degradation of cartilage in this chronic inflammatory disease. Neutrophils are known to generate potent reactive oxygen species including $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$, HOCl and $^1O_2$ and to release lysosomal enzymes (e.g. elastase, collagenase, myeloperoxidase) when they are stimulated (Fantone *et al.*, 1982). Although the contribution of neutrophils (Liszt *et al.*, 1991) and hydrogen peroxide (Roberts *et al.*, 1987) to these pathological conditions is well established, the mechanisms of damage, regulation and defense reactions remain unknown in detail.

We have focused our interest mainly on neutrophil-derived hypochlorous acid because of its strongly oxidizing properties. HOCl is generated in a myeloperoxidase (MPO) catalysed reaction between hydrogen peroxide and chloride anions (Thomas, 1979; Albrich *et al.*, 1981). Myeloperoxidase is present in high concentrations in azurophilic granules of neutrophils and released upon cell stimulation (Klebanoff *et al.*, 1978).

The predominant role of myeloperoxidase and, thus, hypochlorous acid in cartilage degradation is clearly reflected by two facts: First, neutrophils isolated from synovial fluids from patients with rheumatoid arthritis are characterized by a markedly increased native chemiluminescence (Arnhold *et al.*, 1994), which corresponds to a release of reactive oxygen species. Additionally, cell-free synovial fluids from patients with rheumatoid arthritis show substantial ^{1}H-NMR signals for degra-

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dation products of cartilage (N-acetyl groups and acetate). These signal intensities correlate extremely well with the myeloperoxidase activity in the same synovial fluids (Schiller *et al.*, 1996). This fact clearly proves the importance of HOCl in cartilage degradation.

The molecular mechanism of the reaction between hypochlorous acid and cartilage components has been studied in model experiments. HOCl damages N-acetylglucosamine, N-acetylgalactosamine, chondroitinsulfate (CS) and hyaluronic acid (HA) *in vitro*. Two effects have been observed: A breakdown of large polymers, such as hyaluronic acid, into smaller oligomeric units, and a reaction of HOCl with N-acetyl side groups to yield a transient chlorinated product and acetate (Schiller *et al.*, 1994).

Finally, HOCl affects samples of articular cartilage in an analogous manner (Schiller *et al.*, 1995a): glycosidic linkages of polysaccharides are cleaved and low molecular mass components arise. This process can be easily monitored by an increase in ^{1}H-NMR signals at 2.02 ppm for more mobile N-acetyl groups (Grootveld *et al.*, 1991). The second effect is the reaction of HOCl with N-acetyl groups of carbohydrates to yield acetate via a transient chlorinated product. At higher incubation times and higher concentrations of NaOCl formiate is also clearly detectable in these experiments. Interestingly, collagen of cartilage is affected by hypochlorous acid only to a very low extent (Schiller *et al.*, 1995a).

However, in contrast to our and other (Baker *et al.*, 1989; Kowanko *et al.*, 1989) findings, some groups doubted the significance of reactive oxygen species released from neutrophils in glycosaminoglycan degradation of cartilage samples (Moore *et al.*, 1993). Although, these authors stimulated neutrophils with phorbol myristate acetate (PMA), an agent known to induce the generation of reactive oxygen species mainly intracellularly (Dahlgren, 1987), the discussion is rather controverse.

In order to further evaluate effects of hypochlorous acid on carbohydrate degradation, experiments with bovine nasal cartilage and HOCl were performed. Nasal cartilage differs from previously reported articular cartilage in its function and composition. Nasal cartilage has only a pure supporting function and contains about twice as much carbohydrates as articular cartilage and vice versa less collagen (Meyer, 1990; Torchia *et al.*, 1977).

Thus, effects of HOCl should be more expressed towards bovine nasal cartilage due to its enhanced content of carbohydrates.

In principle, HOCl causes the same effects on carbohydrates in nasal cartilage as in articular cartilage. However, the kinetics of changes differ considerably.

Material and Methods

Chemicals

All chemicals (NaCl, Na_2HPO_4 and KH_2PO_4) were purchased from Fluka Feinchemikalien GmbH (Neu Ulm, Germany) of analytical grade and used without any further purification. Double-distilled water was used for preparation of all solutions.

Sodium hypochlorite (as source for hypochlorous acid) was from Sigma Chemie (Deisenhofen, Germany). A stock solution of NaOCl was prepared in water. Its concentration was determined spectrophotometrically at pH 12 (ε_{290}= 350 l $mol^{-1}cm^{-1}$) immediately prior to use (Morris, 1966). A starting concentration of 0.1 mol/l NaOCl was used to obtain final concentrations ranging from 10^{-5} mol/l up to 10^{-2} mol/l sodium hypochlorite.

Cartilage preparation

Fresh bovine nasal cartilage and pig articular cartilage were obtained from juvenile animals (about 12 months old and without any traces of disease) within a few hours after slaughter. After removing the surrounding soft tissue, cartilage was cut into small pieces, frozen in liquid nitrogen and finely minced in a portland mortar to minimize biological deviations.

Incubation conditions

0.5 g (wet weight) of powdered cartilage and 2.0 ml buffer (50 mmol/l phosphate, 0.14 mol/l NaCl, pH 7.4) containing different concentrations of NaOCl were mixed in plastic vessels and incubated at 37 °C in a water bath. For longer incubation periods (more than 2 hours), "merthiolate" (sodium salt of ethyl mercurithiosalicylate) was added at a final concentration of 200 mg/l to pre-

vent bacteria growth (Schiller *et al.*, 1995b). At each sampling time the sample was centrifuged for about 10 minutes at 5000 rev/min to remove insoluble material. The clear, slightly yellow supernatants were analysed by ^{1}H-NMR spectroscopy and in some cases also by ^{13}C NMR spectroscopy. All experiments were at least repeated three times.

NMR-measurements

Proton-NMR measurements were conducted on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^{1}H. All spectra were recorded at ambient temperature (293 K). Typically 0.40 ml supernatant was placed in a 5 mm diameter NMR tube and 50 µl of D_2O was added to provide a field frequency lock. The intense water signal and the broad resonances arising from proteins and polymeric carbohydrates were suppressed by a combination of the Hahn spin-echo sequence (Bell *et al.*, 1987) and presaturation at the water resonance frequency. The Hahn spin-echo sequence [90°-τ-180°-τ-collect] was usually repeated 128 times with τ = 60 ms and an acquisition delay between two pulses of 8 seconds to allow full spin-lattice relaxation (T_1) of the protons in the sample. All spectra were recorded with a spectral width of 4000 Hz (about 13 ppm). No line-broadening or Gauss-broadening was used. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulfonate at a final concentration of 500 µmol/l (Kriat *et al.*, 1992). Resonances were identified by their known chemical shifts and by their subsequent enhancement after addition of a small amount of the corresponding pure compound (acetate and formiate).

^{13}C NMR spectra at 75.47 MHz were obtained on the same spectrometer as described above. Spectra were recorded with a flip angle of 45° (90° flip angle: ~4µs) with a pulse repetition time of 2s (SW 15600Hz/16 K data points). Usually 16 K transients were accumulated under WALTZ-16 decoupling. All free induction decays were processed with a 10 Hz line broadening to improve signal to noise ratio.

Results and Discussion

Cartilage consists of a complex network of different carbohydrate and protein polymers (Meyer, 1990; Torchia *et al.*, 1977) and some water soluble low molecular mass compounds which mainly arise from energy metabolism of cartilage cells, the chondrocytes. Changes in composition of cartilage can be easily monitored in the high resolution NMR spectra of supernatants. Fig. 1 shows typical ^{1}H-NMR spin-echo spectra of supernatants of pig articular cartilage (**a**) and bovine nasal cartilage (**b**) incubated for 2 hours with pure phosphate buffer.

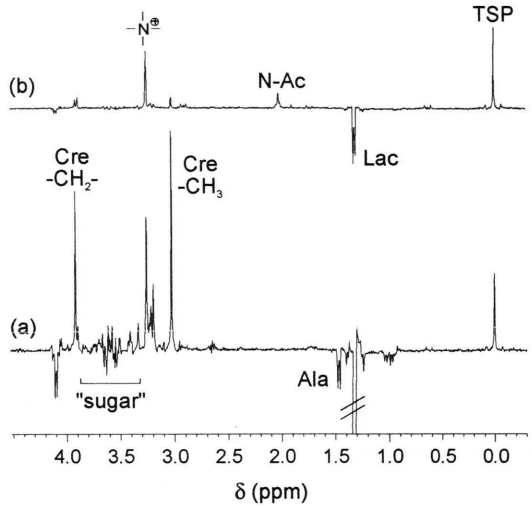

Fig. 1. ^{1}H-NMR spectra of supernatants of pig articular cartilage (0.5 g wet weight) (a) and bovine nasal cartilage (b) after incubation in 2 ml 50 mmol/l phosphate buffer containing 0.14 mol/l NaCl at 37 °C for two hours. The signals at 0.00, 0.63, 1.76 and 2.91 ppm are due to the standard "TSP" (3-(trimethylsilyl)-propane-1-sulfonate). Abbreviations used in peak assignments: Lac, lactate; Ala, alanine; N-Ac, mobile N-acetyl groups; Cre, creatine; N⊕, quarternary ammonium salts.

Most resonances appearing in the spectrum of articular cartilage (**a**) arise from well known, low molecular mass chemical substances (Agar *et al.*, 1991). The spectra are always dominated by a sharp doublet at 1.31 ppm (cut off for clarity) arising from extracellular lactate, which can also be found in most tissues and body fluids like human plasma (Nicholson *et al.*, 1984), cerebrospinal fluid (Petroff *et al.*, 1986) and synovial fluids (Schiller *et al.*, 1996; Parkes *et al.*, 1991). A further resonance for lactate is found at 4.09 ppm, showing a quartet for the C-H group. The protons from the -OH and the -COOH group are not visible in the spectra because of their fast exchange with water molecules.

All other metabolic species given in Fig. 1a are by far less intense than the lactate signal. There are singlets for creatine (δ=3.90 ppm for the CH_2 group and δ=3.00 ppm for the CH_3 group), quarternary ammonium salts (3.19 and 3.25 ppm) and a weak doublet for alanine (δ=1.46 ppm) (Agar *et al.*, 1991). Mobile N-acetyl groups of cartilage polymers (δ~2.0 ppm) (Schiller *et al.*, 1996; Grootveld *et al.*, 1991), valine (δ=0.97 and 0.93 ppm) and glucose (δ~3.3–3.8 ppm) are present only in low amounts after incubation for two hours.

In comparison to articular cartilage, nasal cartilage (Fig. 1b) contains less resonances and especially to a less extent metabolites as creatine (Cre) and lactate (Lac). Only components containing quarternary ammonium groups (e.g. choline and betaine) are present in comparable concentrations.

However, significant differences are found in the 2.0 ppm region due to the different composition of both kinds of cartilages (Torchia *et al.*, 1977). The relatively small and broad resonance at 2.0 ppm indicates that nasal cartilage contains much more low molecular mass glycosaminoglycans, showing a resonance at this frequency according to their relatively mobile N-acetyl groups. Whereas nasal cartilage contains about as twice as much glycosaminoglycans than articular cartilage (Torchia *et al.*, 1977), it contains only about the half of collagen in comparison to articular cartilage (Meyer, 1990).

High signals at 2.02 ppm for N-acetyl groups in bovine nasal cartilage after incubation with phosphate buffer seem to be attributable mainly to chondroitinsulfate. This polysaccharide with a molecular mass lower than 50000 Da yields intense signals at 2.02 ppm (Schiller *et al.*, 1994). Bovine nasal cartilage contains higher amounts of chondroitinsulfate than articular cartilage (Meyer, 1990). A fast release of chondroitinsulfate after incubation with buffer seems to occur in our experiments.

The resonance at 2.0 ppm is a very useful marker towards changes in the molecular weight of cartilage carbohydrates (Schiller *et al.*, 1996; Grootveld *et al.*, 1991; Parkes *et al.*, 1991).

Upon the addition of the strong oxidizing reagent, sodium hypochlorite, some characteristic changes occur in the ^{1}H-NMR spectra of bovine nasal cartilage depending on the actual concentration of NaOCl and the incubation time. Regions of interest of the proton-NMR spectra of bovine nasal cartilage after incubation at 37 °C with pure buffer (a), 10^{-4} mol/l (b), 10^{-3} mol/l (c) and 10^{-2} mol/l (d) NaOCl for two hours are selected in Fig. 2.

The doublet at the high field end of the spectra corresponds to the methyl group of alanine (δ= 1.46 ppm), which is an important metabolite in different kinds of body fluids. As it is clearly visible from the spectra this resonance is diminished effi-

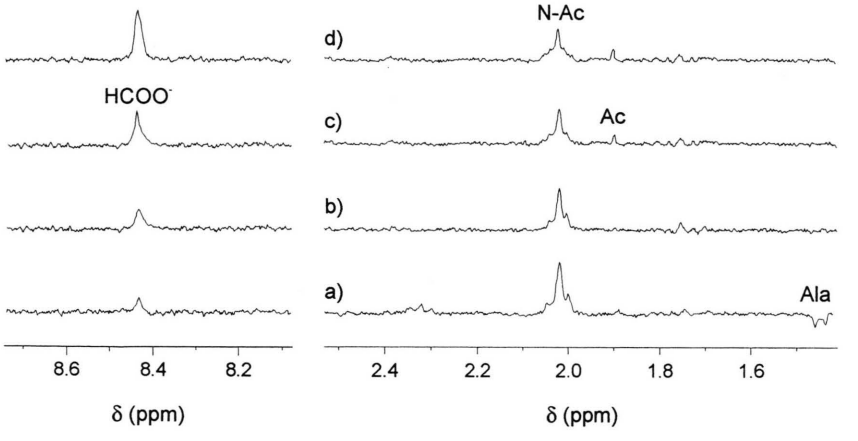

Fig. 2. ^{1}H-NMR spectra of bovine nasal cartilage after incubation for two hours in 2 ml 50 mmol/l phosphate buffer containing different amounts of NaOCl: 0 mol/l (a), 10^{-4} mol/l (b), 10^{-3} mol/l (c) and 10^{-2} mol/l NaOCl (d). The up and the down field region of the spectra have been separated for clarity. Abbreviations used in peak assignments: Ala, alanine; N-Ac, mobile N-acetyl groups; Ac, acetate.

ciently by the reaction with sodium hypochlorite due to a chloramine formation. Already at a 10^{-4} mol/l NaOCl concentration no more alanine is detectable in the NMR spectra.

However, most important changes occur in the 2.0 ppm region of the proton NMR spectra. The intense resonance at 2.02 ppm in the absence of NaOCl (**2a**) is clearly diminished by the action of sodium hypochlorite under the formation of formiate. Formiate is believed to be a potent indicator for carbohydrate degradation (Grootveld *et al.*, 1991).

Interestingly, the broad resonance at about 2.0 ppm seems to be split into three different resonances. This is caused by different structures of sugar molecules in carbohydrate polymers. For example, chondroitinsulfate yields neighboured resonances at 2.005 and 2.020 ppm for chondroitin-4- and chondroitin-6-sulfate (Welti *et al.*, 1979).

It has been shown recently that NaOCl reacts also with N-acetyl groups in carbohydrates (Schiller *et al.*, 1994) to yield new resonances at 1.90 ppm for acetate and at 2.35 ppm for a transient chlorinated product. The former has been identified in solutions of N-acetylglucosamine and chondroitinsulfate treated with NaOCl (Schiller *et al.*, 1994). A small peak for acetate is formed in nasal bovine cartilage only using high concentrations of NaOCl, whereas no signals for the expected transient product appear at 2.35 ppm.

An additional marker for cartilage degradation is not observed in the up-field part of the spectra, but in the down-field region. Only a small resonance at 8.44 ppm is found in the absence of NaOCl, but a steadily increasing signal is observed with increasing concentrations of NaOCl. This resonance is assigned to the single formiate proton, which is assumed to be a potent indicator for the complete degradation of the carbohydrate skeleton. It has been shown that the exposure of carbohydrate solutions to γ-irradiation leads to the formation of formiate (Grootveld *et al.*, 1991). Although the formation of formiate in these experiments is caused by hydroxyl radicals, formiate is also formed upon the action of NaOCl in high concentrations (Schiller, 1995c). Formiate is also present in low concentrations in pathologically changed synovial fluids (Grootveld *et al.*, 1991).

To investigate the formation of acetate, nasal cartilage was also treated with a very high concentration of NaOCl (100 mmol/l). Here, more complex changes were observed especially in the ^{1}H-NMR spectrum (Fig. 3). There are three predominant resonances in the 2.0 ppm region. These signals contribute to relatively mobile N-acetyl side chains of polysaccharides (~2.0 ppm), a transient product formed by the reaction between sodium hypochlorite and the N-acetyl side groups of polysaccharides (~2.35 ppm) (Schiller *et al.*, 1994) and to acetate (1.90 ppm). These changes are in very good accordance with the effects obtained by the action of HOCl on polysaccharide solutions.

Additionally, the collageneous network of cartilage is also affected by these high concentrations of hypochlorous acid, as visible by the relatively broad resonances at about 3.70 and 0.85 ppm, which can be attributed to fragments of collagen with rather high mobility (Schiller, 1995c).

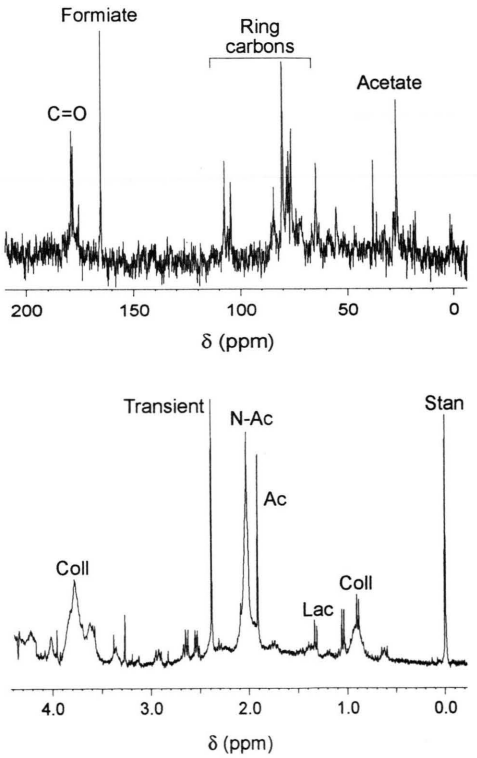

Fig. 3. ^{1}H- and ^{13}C-NMR spectra of bovine nasal cartilage after incubation for two hours in 2 ml 50 mmol/l phosphate buffer containing 0.1 mol/l NaOCl. Abbreviations used in peak assignments: N-Ac, mobile N-acetyl groups; Ac, acetate; Lac, lactate; Coll, collagenous fragments; Stan, standard.

Furthermore, the lactate concentration is considerably diminished and formiate is formed in high amounts (data not shown). Additionally, there are some narrow, well resolved resonances at about 0.90, 1.05, 2.55 and 2.63 ppm, which are not present when lower concentrations of HOCl are used. These resonances could not be assigned.

Unfortunately, due to its very low sensitivity compared to proton-NMR spectroscopy (Albert *et al.*, 1993; Schiller *et al.*, 1995c), carbon NMR spectroscopy (**Fig. 3**) does not offer further advantages over the easily accessible used proton-NMR spectra. In the corresponding ^{13}CNMR spectrum of bovine nasal cartilage incubated with 100 mmol/l sodium hypochlorite only a few well-resolved resonances are detectable. Besides resonances for the C-H carbon atoms of different low-molecular mass carbohydrates which cannot be exactly assigned, there are only a few resonances which correspond to low molecular mass degradation products of carbohydrates. Acetate (27.2 ppm) and formiate (163.3 ppm) are clearly visible in the spectrum, indicating a breakdown of N-acetyl side chains (acetate) and a breakdown of the carbohydrate skeleton (formiate) (Schiller *et al.*, 1994; Schiller *et al.*, 1995c). The relatively broad resonance at 178 ppm arises from a mixture of carbonyl groups of N-acetyl groups of cartilage polymers. This resonance is much more expressed after incubation of cartilage with sodium hypochlorite than with pure phosphate buffer (data not shown).

To better understand effects of hypochlorous acid, changes in concentration of N-acetyl groups and acetate in supernatants of bovine nasal cartilage were investigated as a function of time. Fig. 4 shows the time-effect curves for the acetate and N-acetyl group concentration after incubation of bovine nasal cartilage with pure phosphate buffer and 10 mmol/l NaOCl.

Whereas there are only very low amounts of acetate after incubation of cartilage with pure buffer, its concentration is about five- to sixfold enhanced in the presence of NaOCl due to the cleavage of the N-acetyl side chains induced by HOCl. The concentration of acetate increases during the first two hours of incubation with a fast increase in the first 30 minutes followed by a more slower one. Then the acetate concentration remains nearly constant. After 18 hours of incubation the acetate concentration does not exceed 250 μmol/l.

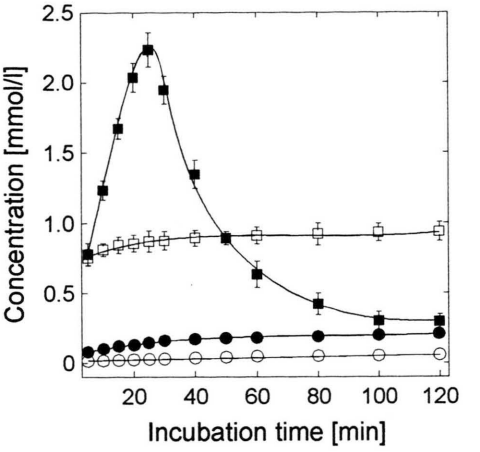

Fig. 4. Molar concentrations of acetate (●, ○) and N-acetyl groups (■, □) after incubation of bovine nasal cartilage with phosphate buffer in the presence (filled symbols) or absence (empty symbols) of 10^{-2} mol/l NaOCl.

This is a marked difference to investigations on articular cartilage. In this case the acetate concentration increases continuously. This difference is possibly caused by the different cartilage compositions. A different behaviour is additionally found for the content of ^{1}HNMR-detectable N-acetyl groups in bovine nasal cartilage.

In the presence of pure buffer there is a rather high concentration of N-acetyl groups, which does not show a significant dependence on incubation time, i.e. their concentration remains nearly the same even at longer incubation times. This can be interpreted as a fast release of polymeric carbohydrates from the collagen network in cartilage, which is already complete after a few minutes in the presence of buffer. However, this behaviour is markedly changed in the presence of NaOCl.

A drastic increase in integral signal intensities at 2.02 ppm occurs during the first 25 minutes of incubation. Then the content of N-acetyl groups decreases markedly to reach a final concentration at about 250 μmol/l. This value is three to four fold lower than the control.

This reaction behaviour clearly indicates marked differences to the behaviour of pig articular cartilage. First of all, a different time course of events was observed. In both kinds of cartilage the intensity maximum for N-acetyl groups was observed at different incubation times, at 90 minutes for articular (Schiller *et al.*, 1995a) and 25 minutes

for nasal cartilage . All changes were finished after two hours of incubation with NaOCl in bovine nasal cartilage, whereas changes in N-acetyl groups and acetate in pig articular cartilage were detected even at longer times. Additionally, both kinds of cartilage differed considerably in signal intensities for N-acetyl groups in incubation experiments with pure phosphate buffer. This different behaviour is assumed to be caused by the tighter collagen network in pig articular cartilage.

Hypochlorous acid causes also the formation of acetate from N-acetyl groups in bovine nasal cartilage. However, much higher values would be expected for acetate if changes in absolute levels of N-acetyl groups were compared to concentrations of acetate formed. The reason for this discrepancy could not be found. Other oxidation processes leading to the formation of non detectable products like carbon dioxide and water could not be excluded. Another possibility is the presence of processes degrading acetate in our cartilage samples.

Nevertheless and despite of open questions, we conclude that hypochlorous acid is massively involved in the pathogenesis of rheumatoid arthritis, because only the involvement of this reactive oxygen species is able to explain all experimental data.

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