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ORIGINAL ARTICLE

Tetsunao Matsushita · Kanji Fukuda
Hironori Yamamoto · Kenji Yamazaki
Takashi Tomiyama · Masamichi Oh · Chiaki Hamanishi

Effect of ebselen, a scavenger of reactive oxygen species, on chondrocyte metabolism

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Abstract Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a synthetic heterocyclic seleno-organic compound, has been shown to act as a scavenger of reactive oxygen species (ROS). We have previously reported that interleukin-1 (IL-1) inhibited proteoglycan (PG) synthesis and induced the production of ROS in cartilage explants and isolated chondrocyte cultures. In this study, we report the protective effect of ebselen against IL-1-mediated inhibition of PG synthesis and ROS induction in cultured cartilage explants and chondrocytes. Ebselen also reversed the inhibition of PG synthesis in mechanically stressed cultured chondrocytes. These data suggest that the use of the antioxidant ebselen may be a useful tool for studying the mechanisms of cartilage degradation.

Key words Chondrocyte · Ebselen · Interleukin-1 (IL-1) · Nitric oxide · Peroxynitrite

Introduction

Articular chondrocytes, which are differentiated cells embedded in an avascular matrix, were exposed to low partial pressures of oxygen and exhibited predominantly anaerobic metabolism. In arthritis, there is a structural and functional degradation of the articular cartilage, the mechanisms of which have received much attention. We have previously shown that reactive oxygen species (ROS), such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), are involved in the cartilage degradation associated with inflammatory joint disease.[1–4]

T. Matsushita · K. Fukuda (✉) · H. Yamamoto · K. Yamazaki · T. Tomiyama · M. Oh · C. Hamanishi
Department of Orthopaedic Surgery, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-sayama 589-8511, Japan
Tel. +81-723-66-0221; Fax +81-723-66-0206
e-mail: k-fukuda@med.kindai.ac.jp

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a synthetic heterocyclic seleno-organic compound, has been shown to be a glutathione peroxidase-mimetic[5] and to act as a scavenger of ROS.[6]

Interleukin-1 (IL-1), a catabolic cytokine that is capable of inducing cartilage breakdown both in vivo[7] and in vitro,[8] is a key mediator of arthritis.[9] We previously showed that IL-1 inhibited proteoglycan (PG) synthesis[10] and induced ROS in cartilage explants and chondrocyte cultures.[4] In this study, we investigated whether ebselen exerts a protective effect against these IL-1-mediated effects in cartilage explants and chondrocyte cultures.

Although mechanical stress is known to be an essential factor in the regulation of cartilage metabolism, the precise mechanisms involved have not yet been determined.[11] To examine the underlying mechanisms leading to cartilage degradation, we introduced a computerized, pressure-operated device (Flexercell Strain Unit) to induce the cyclic tensile stretch of chondrocytes. We have reported that cyclic tensile stretch loaded on chondrocytes inhibited PG synthesis,[12] but the mechanism of this action is not yet understood.

The goal of this study was to determine the effect of ebselen on IL-1-inihibited PG synthesis and IL-1-induced ROS synthesis in cartilage explants and chondrocyte cultures. In addition, we also examined the effect of ebselen on stress-inhibited PG synthesis in chondrocyte cultures.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Human recombinant IL-1-β was purchased from R&D systems (Minneapolis, MN, USA). Cetylpyridinium chloride, human recombinant superoxide dismutase (SOD), and ebselen were purchased from Sigma (St. Louis,

MO, USA). [^{35}S]-sulfate was obtained from New England Nuclear (Boston, MA, USA). 2,7-dichlorofluorescin diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA).

Chondrocyte culture and PG synthesis

Bovine radiocarpal joints were obtained from a local slaughterhouse. One joint usually yielded a sufficient number of articular chondrocytes to carry out one series of experiments. Chondrocytes, enzymatically released as described elsewhere,[13] were seeded at a density of 2×10^5 cells/ml in 24-well plates (Becton Dickinson, NJ, USA) and cultured for 5 days at 37°C in an atmosphere of 95% air and 5% CO_2. The culture medium was DMEM (1 ml/well) containing 10% heat-inactivated FBS, and penicillin/streptomycin/amphotericin B (100 IU/ml, 100 μg/ml, and 0.25 μg/ml, respectively), and was changed every 2 days.

Organ culture

Full-thickness explants of articular cartilage were taken from bovine carpal joints obtained from a local slaughterhouse. The cartilage was cut into uniform disks. These discs were weighed and placed into a 24-well culture dish. The culture medium was DMEM (1 ml/well) containing 10% heat-inactivated FBS. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. To achieve steady-state metabolism, the disks were precultured for 7 days and then the medium was replaced with serum-free conditions, with or without IL-1 α/β (10 ng/ml) and ebselen (1 μM).[14]

Alginate bead culture

Bovine articular chondrocytes, isolated as described above, were suspended in 1.2% alginate in 0.9% NaCl at a density of 4×10^6 cells/ml, and then passed drop-wise through a 22-gauge needle into 100 mM $CaCl_2$ solution. After 10 min polymerization, the beads were washed three times in 0.9% NaCl and once in DMEM medium with 10% FBS.[15]

Confocal microscope

Samples (cartilage explants and alginate beads) were incubated with 50 μM DCF-DA in Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 15 mM HEPES, 10 mM glucose, pH 7.3) for 15 min, and then washed with the same solution. These samples were examined with a laser scanning confocal microscope (MRC 2400 LSX imaging system; Bio-Rad, Hertfordshire, UK) equipped with an argon laser adjusted to an output of 250 mW at 488 nm excitation, 525 nm emission.

Fluorescent measurement

Monolayer chondrocytes were cultured in the absence or presence of IL-1 (10 ng/ml) in DMEM medium with 10% FBS for the time indicated in 24-well plates. In separate experiments, SOD (100 IU/ml), L-N-monomethylarginine (L-NMA, 1 mM), or ebselen (1 μM) were added simultaneously with IL-1. The culture medium was replaced by Locke's solution containing DCF-DA, as described above. Fluorometric measurement of the DCF oxidation in the cell layers was carried out using confocal microscopy. The data obtained were quantitatively analyzed and expressed as pixel count using LaserSharp Processing software. Six replicates per treatment group were run for each experiment.

Proteoglycan (PG) synthesis

On day 6 the medium was replaced with freshly prepared DMEM containing IL-1 (10 ng/ml) and ebselen (1 μM), and the cells were cultured for 24 h. After 20 h of this incubation period, [^{35}S]-sulfate (0.74 KBq/ml) was added to the culture medium for the final 4 h of incubation. The media were collected, and the cell monolayers were washed three times with ice-cold phosphate-buffered saline (PBS). Using a rubber policeman, the cells were then put into 1 ml PBS and sonicated for 30 s at 0°C. The content of newly synthesized PG present within the media and chondrocytes was measured by assessing the incorporation of [^{35}S]-sulfate into cetylpyridinium chloride (CPC) perceptible material.[16] Six replicates per treatment group were run for each experiment.

In vitro application of stretch

Monolayer chondrocytes were subjected to mechanical stretch using a vacuum-operated instrument (FX-3000, Flexercell Strain Instrument, Flexcell Corp.) The frequency and degree of elongation of the deformable substrate were computer-controlled. According to the supplier's manual, there is a linear relationship between the vacuum level (kPa) and the maximal percentage elongation of cells. After the deformable substrate plates were placed in the vacuum-operated instrument, the chondrocytes were stretched repeatedly for 3 s and then relaxed. The regimen was set at 10 cycles per minute, i.e., every 6 s, the chondrocytes were stretched for 3 s with 10 kPa of stress.[12] The culture medium was MEM, supplemented with penicillin–streptomycin–fungizone and 10% FBS at 37°C in a 5% CO_2 environment. The chondrocytes were stretched for 24 h. After 20 h of this incubation period, [^{35}S]-sulfate (0.74 KBq/ml) was added to the culture medium for the final 4 h of incubation.

Statistical analysis

Results are presented as the mean ± SD. Significant differences were determined by Student's *t*-test. A significance level of $P < 0.05$ was used to reject the null hypothesis.

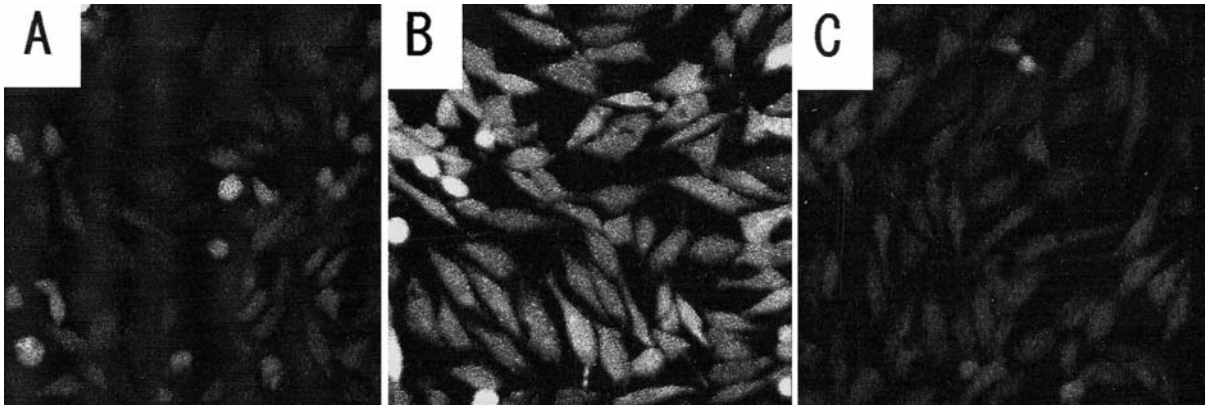

Fig. 1. DCF-oxidation with IL-1 treatment. Monolayer chondrocytes were treated with IL-1 (10 ng/ml) in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal bovine serum (FBS) for 6 h, and then the medium was changed to a serum-free medium containing the fluorescent probe 2,7-dichlorofluorescin (DCF-DA) (5 μM). The desired depth of the cell was obtained with visible light after incubation with DCF-DA by confocal microscopy and excited using an argon laser. Chondrocytes were incubated in the absence (**A**) or presence (**B**) of IL-1 (10 ng/ml). When superoxide dismutase (SOD) (100 IU/ml) was added to the medium, the IL-1-induced DCF-oxidation was inhibited (**C**). ×200

Results

In monolayer chondrocytes cultured with or without IL-1 for 6 h prior to the addition of DCF-DA, confocal microscopy revealed an extensive DCF-oxidation in the cytoplasm of each cell. Negligible or scanty DCF-oxidation was noted in untreated chondrocytes. When SOD, which accelerates the dismutation reaction of superoxide, was added simultaneously with IL-1, the IL-1-enhanced DCF-oxidation was inhibited (Fig. 1). NMA, which inhibits NO synthesis, also inhibited IL-1-enhanced DCF-oxidation (data not shown).

We then examined the time course of DCF-oxidation with IL-1 treatment. Monolayer chondrocytes were incubated with IL-1 for different time periods, and fluorimetric measurements of DCF-oxidation were determined using confocal microscopy. The significant enhancement of DCF-oxidation in IL-1-treated chondrocytes compared with untreated chondrocytes specifically began after 3 h and peaked 2–3 h later, when an almost two-fold increase was recorded. In the presence of ebselen, IL-1-enhanced DCF-oxidation was completely abolished (Fig. 2).

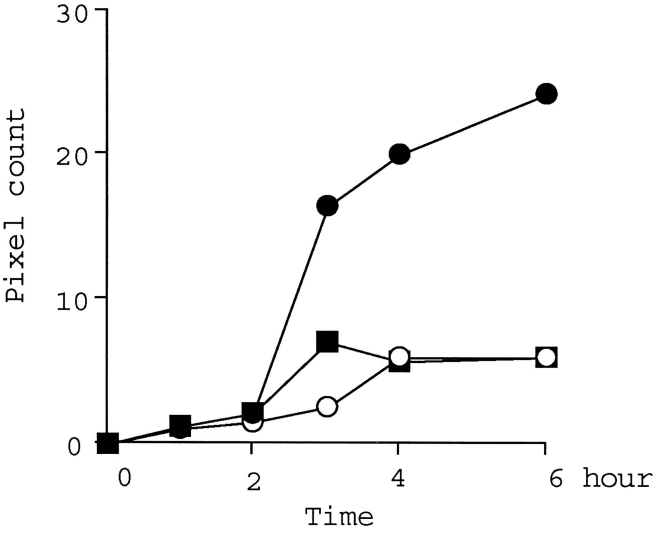

Fig. 2. Time-course for IL-1-induced DCF-oxidation. Chondrocytes were incubated with IL-1 in DMEM medium with 10% FBS for different time-periods, and then the medium was changed to a serum-free medium containing the fluorescent probe DCF-DA (5 μM). Using confocal microscopy, fluorimetric measurement of DCF-oxidation was carried out. Representative results of three different experiments are shown. *Open circles*, control; *solid circles*, IL-1 (10 ng/ml); *solid squares*, IL-1 (10 ng/ml) + ebselen (1 μM). The means of six wells are plotted. Each standard deviation was less than 15% of the mean value. $^{**}P < 0.01$

To confirm the effect of ebselen on IL-1-enhanced DCF-oxidation, two separate approaches were taken, cartilage explant culture and the alginate bead culture system. The cartilage explants were incubated with or without IL-1 for 5 h prior to the addition of DCF-DA. Confocal microscopy revealed negligible or scanty DCF-oxidation in the untreated cartilage. However, IL-1 treatment led to extensive DCF-oxidation in the cytoplasm of each chondrocyte. Ebselen, added simultaneously with IL-1, inhibited IL-1-enhanced DCF-oxidation (Fig. 3). As with cartilage explants, chondrocytes embedded in alginate beads showed an IL-1-enhanced DCF-oxidation that was inhibited by ebselen (Fig. 4).

In monolayer chondrocyte cultures, the presence of IL-1 resulted in an approximately 60% inhibition of PG synthesis when compared with untreated chondrocytes. This IL-1-inhibited PG synthesis was reversed by ebselen (Table 1). To determine the precise effect of ebselen on IL-1-inhibited PG synthesis, different concentrations of ebselen were added in this system. Ebselen reversed IL-1-inhibited PG synthesis in a dose-dependent fashion (Fig. 5).

We also examined the effect of ebselen on the mechanical stress-mediated alteration of PG synthesis. When cyclic tensile stretch was loaded in the presence of ebselen (1 μM), the stretch-inhibited PG synthesis was completely recovered (Fig. 6).

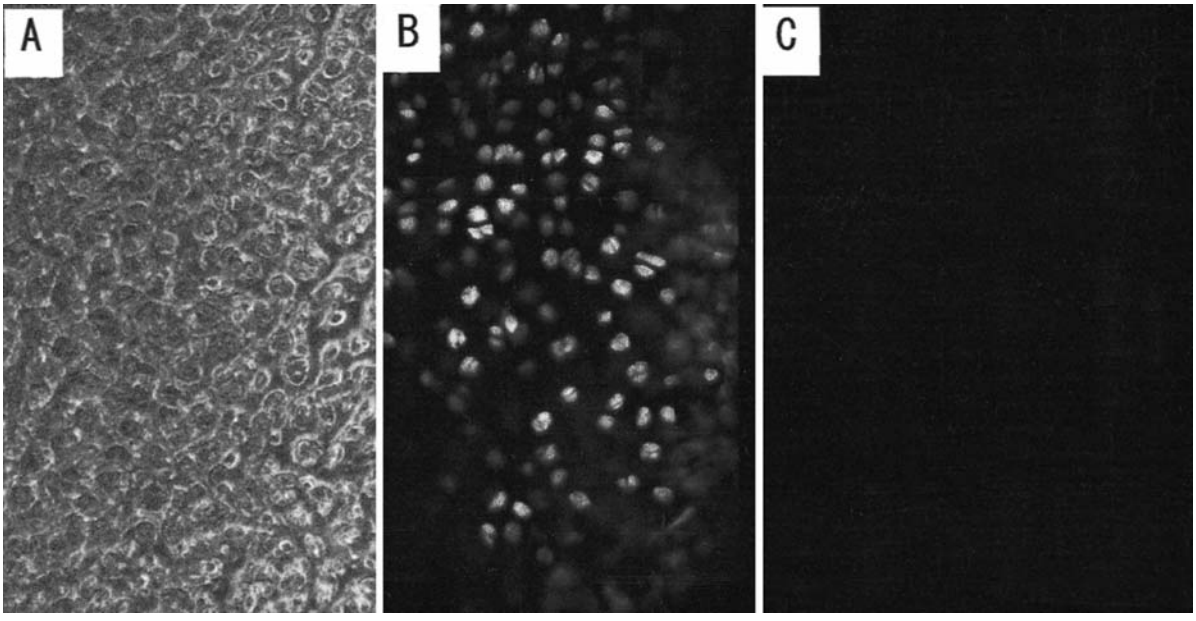

Fig. 3. IL-1-induced DCF-oxidation in articular cartilage explants. Cartilage slices were cultured in the absence or presence of IL-1 (10 ng/ml) in DMEM medium with 10% FBS for 5 h. The medium was then changed to a serum-free medium containing the fluorescent probe DCF-DA (5 μM) for 15 min. The desired depth of the cartilage slice was obtained with visible light after incubation with DCF-DA by confocal microscopy (**A**), and excited using an argon laser (**B**). When ebselen (1 μM) was added to the medium, the IL-1-induced DCF-oxidation was completely inhibited (**C**)

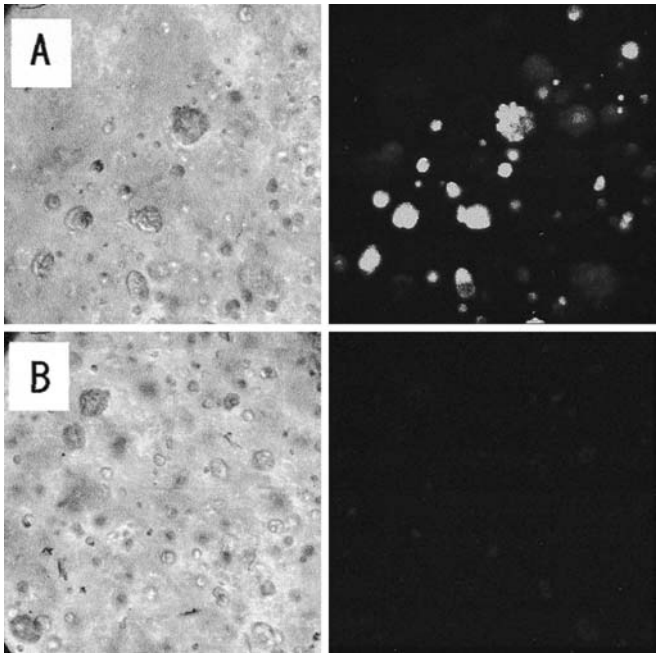

Fig. 4. IL-1-induced DCF-oxidation in chondrocytes embedded in alginate beads. Tissues were cultured in the absence or presence of IL-1 (10 ng/ml) for 6 h. The desired depth of tissue slice was obtained by confocal microscopy (*left lane*) and excited using an argon laser (*right lane*). **A**, IL-1 (10 ng/ml); **B**, IL-1 (10 ng/ml) + ebselen (1 μM). ×200

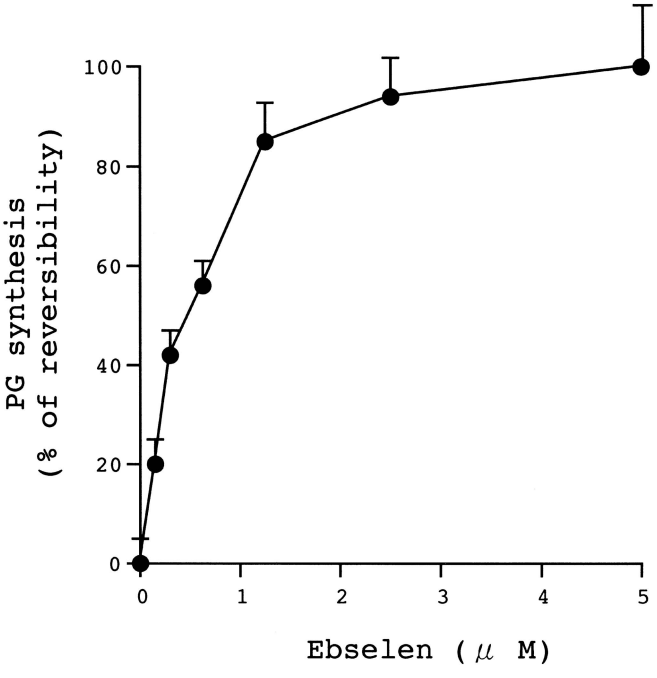

Fig. 5. Effect of ebselen on IL-1-inhibited proteoglycan (*PG*) synthesis of the chondrocytes. Cells were cultured in the absence or presence of IL-1 (10 ng/ml) for 24 h. Different concentrations of ebselen were added and proteoglycan (PG) synthesis was measured. Data are expressed as mean ± SD of the percentage of reversible against IL-1-inhibited PG synthesis. $n = 6$

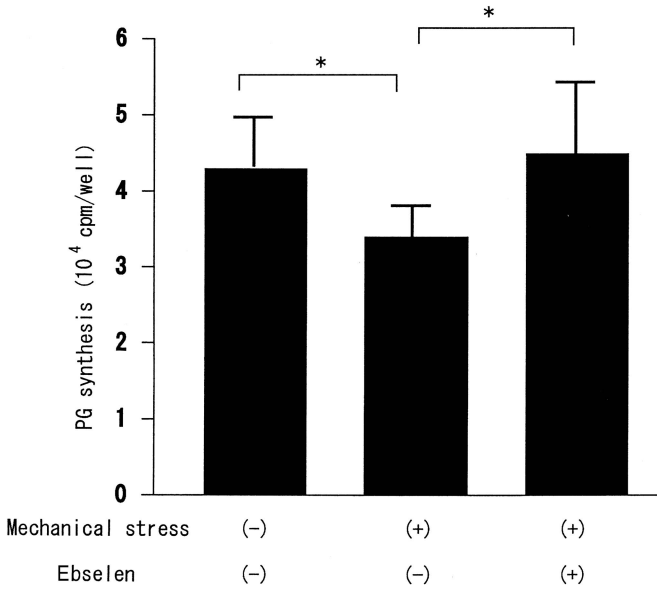

Fig. 6. Effect of ebselen on mechanical stress-mediated PG synthesis of chondrocytes. The cyclic tensile stretch (mechanical stress) was loaded on the monolayer chondrocytes in the absence or presence of 1 μM ebselen. Proteoglycan (PG) synthesis was expressed as 10^4 cpm/well. Data are expressed as mean ± SD. $n = 6$; $^*P < 0.01$

Table 1. Effect of ebselen on IL-1-inhibited PG synthesis

	Exp. 1	Exp. 2
Control	1.56 ± 0.14	1.70 ± 0.13
IL-1	1.06 ± 0.11*	0.95 ± 0.19*
IL-1 + ebselen	1.40 ± 0.09**	1.30 ± 0.07**

Cells were cultured in the absence or presence of agents for 24 h. Concentrations used were IL-1 (10 ng/ml), ebselen (1 μM). Proteoglycan (PG) synthesis was expressed as 10^4 cpm/well. Data were expressed as mean ± SD of six determinations
* $P < 0.05$ versus control; ** $P < 0.05$ versus IL-1

Discussion

DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to a nonfluorescent derivative (2′,7′-dichlorofluorescin) that is trapped within the cells. In the presence of intracellular ROS, this compound is oxidized to the highly fluorescent compound DCF.[17] In this study, we used DCF-DA to detect the in situ induction of ROS by chondrocytes. When SOD, which accelerates the dismutation reaction of superoxide anion into hydrogen peroxide, was added simultaneously with IL-1, IL-1-enhanced DCF-oxidation was inhibited. These data suggest that IL-1 induced ROS, including the superoxide anion, and SOD neutralized ROS in the cells. However, direct evidence showed that exogenously added SOD penetrates into the chondrocytes.

We also found that IL-1 induced extensive DCF-oxidation in the cytoplasm of monolayer chondrocytes, viable cartilage slices, and chondrocytes embedded in alginate beads. One of the possible ROS which reacts with DCF-DA is peroxynitrite ($ONOO^-$), which is formed from the reaction $O_2^- + NO \rightarrow ONOO^- + H^+$. Recent studies showed that DCF-DA is an excellent marker with the potential to detect peroxynitrite formation in living cells.[17] When IL-1 stimulates NO synthesis via an inducible form of NO synthase (iNOS), a 5-h incubation was necessary to induce iNOS mRNA in bovine chondrocytes.[18] The kinetics studies reported here showed that a 3 h lag time was necessary to induce DCF-oxidation. We considered that the NO that reacted with the superoxide anion to form peroxinitrite is both constitutive NO and inducible NO. Although the exact role of NO is not fully understood, constitutive NO produced in the chondrocytes may play an important role in chondrocyte metabolism.

Ebselen, a synthetic selenium-containing compound, reacts efficiently with peroxynitrite and exhibits a potent antioxidant action.[5,6] Its anti-inflammatory properties have already been reported.[19,20] In 1998, Pratta et al.[21] reported the effect of ebselen on IL-1-induced alterations in cartilage metabolism. Although they demonstrated the inhibitory effect of ebselen on IL-1-enhanced cartilage degradation, they failed to demonstrate the protective effect on IL-1-inhibited PG synthesis. This discrepancy between their results and ours could be because of the source of the cartilage; they used bovine nasal cartilage and we used bovine articular cartilage. The concentration of IL-1 (500 ng/ml) that Pratta et al. used was also extremely high when compared with our concentration (10 ng/ml). They also did not show any effects of ebelsen on the induction of ROS in IL-1-treated chondrocytes. To the best of our knowledge, our report is the first to demonstrate the protective effect of ebselen against IL-1-evoked cartilage ROS generation.

Extensive studies have demonstrated that ebselen is able to protect the brain against ischemic injury in both human clinical trials and various animal models.[22] Ebselen has also been shown to prevent neurotoxicity and clinical symptoms in a primate model of Parkinson's disease.[23] The mechanism underlying the neuroprotection afforded by ebselen is still not completely understood, but it is most certainly related to its antioxidant and anti-inflammatory properties.

In this context, the reversible effect of ebselen on mechanical stress-inhibited PG synthesis is interesting. In this study, when we loaded cyclic tensile stretch on monolayer chondrocytes, the cyclic tensile stretch inhibited PG synthesis, which is consistent with previous work.[12] However, ebselen reversed these inhibitory actions. These data suggest the involvement of ROS in mechanical stress-mediated cartilage degradation. There are many explanations for this phenomenon. Fujisawa et al.[24] reported an enhanced production of IL-1 with cyclic tensile stretch using chondrocytes. It is well accepted that chondrocytes possess a NADPH oxidase and that they produce ROS.[25] It is possible that ROS production, stimulated by IL-1, is also enhanced by the applied cyclic tensile stretch. The induced ROS, in turn, inhibits the PG synthesis of the chondrocytes. Although further studies are necessary to determine the precise mechanisms of ROS production with mechanical stress, the use of the antioxidant ebselen is a useful new tool

to determine the mechanisms of stress-mediated cartilage degradation.

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