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Endoplasmic reticulum stress mediates nitric oxide-induced chondrocyte apoptosis

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Abstract. Nitric oxide (NO) is one of the most important mediators of chondrocyte apoptosis, which is a notable feature of cartilage degeneration. While apoptosis of chondrocytes is induced by p53, NO can also induce endoplasmic reticulum (ER) stress, which may be involved in the process of NO-induced chondrocyte apoptosis. The aims of this study were to determine whether NO-induced ER stress (ERS) leads to apoptosis of chondrocytes and to investigate the temporal relationship between the expression of C/EBP-homologous protein (CHOP), an ERS-associated apoptotic molecule, and the expression of p53 during apoptosis in NO-stimulated chondrocytes. Rat chondrocytes were stimulated by sodium nitroprusside (SNP), a NO donor. Real-time polymerase chain reaction (PCR) was performed to analyze the mRNA expression of CHOP, glucose-regulated protein (GRP78) and p53. Apoptosis of chondrocytes was quantified using an enzyme-linked immunosorbent assay (ELISA). SNP-treated chondrocytes showed an increase in CHOP and GRP78 mRNA expression and underwent apoptosis. Sodium 4-phenylbutyrate (PBA), an ERS inhibitor, reduced CHOP and GRP78, as well as SNP-stimulated apoptosis of chondrocytes, without affecting the SNP-dependent generation of NO. In addition, the blockade of CHOP following siRNA transfection reduced SNP-induced apoptosis of chondrocytes. The CHOP expression increased after apoptosis was detected in the SNP-treated chondrocytes, whereas the p53 expression increased prior to apoptosis. These data demonstrated that NO-induced ERS leads chondrocytes to apoptosis, although this effect appears to be limited to persistent impairment of NO stimulation. These findings may provide insight into the pathology of cartilage degeneration.

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Introduction

Nitric oxide (NO) is a significant inducer of chondrocyte apoptosis, a feature of cartilage degeneration in diseases such as osteoarthritis (OA) (1-3). NO-stimulated chondrocytes increased the expression of tumor suppressor p53 via phosphorylation of the p38 mitogen-activated protein kinase, resulting in the enhanced transcription of Bax, a pro-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family (4,5). Accumulation of Bax in the mitochondria leads to cell apoptosis through the release of cytochrome *c* from the mitochondria (4). Studies showing that the reduction of the p53 expression inhibits NO-induced apoptosis of chondrocytes provide additional evidence of p53 participation in such apoptosis (4,5).

Endoplasmic reticulum (ER) stress, provoked by an imbalance between the load of unfolded proteins in the ER and the capacity of the ER, leads to the accumulation of unfolded or misfolded proteins in the ER (6). Mammalian cells induce specialized responses to recover or maintain ER function by attenuating general translation, upregulating the expression of ER chaperone proteins, such as the 78 kDa glucose-regulated protein (GRP78), and by activating the endoplasmic reticulum-associated protein degradation system (6). However, if these protective responses fail and ER stress (ERS) persists, specialized apoptotic pathways, such as the enhanced expression of C/EBP-homologous protein (CHOP) and the activation of ER-associated caspase 12, are activated to eliminate the damaged cells (7). Previous studies showed that articular chondrocytes in OA cartilage experience ERS during cartilage degeneration (8,9). ERS induced by pharmacological ERS inducers such as tunicamycin and thapsigargin may induce apoptosis of chondrocytes (10). Previously, we demonstrated that the number of chondrocytes exhibiting ERS correlates with the number of apoptotic chondrocytes in osteoarthritic cartilage (11). Thus, ERS is important in chondrocyte apoptosis during the process of cartilage degeneration.

In their study, Oliver *et al* (12) showed that NO is a potent inducer of ERS in chondrocytes. However, NO has not been demonstrated to induce sufficient ERS to induce apoptosis in chondrocytes. In addition, little is known about the correlation between the ERS- and p53-mediated apoptotic pathways induced by NO in chondrocytes.

The objective of this study was to determine whether NO-induced ERS leads to apoptosis in chondrocytes by exam-

ining whether NO-induced apoptosis in cultured chondrocytes was suppressed by attenuating ERS using the chemical chaperone ERS inhibitor sodium 4-phenylbutyrate (PBA) or by blocking the ERS-associated apoptotic pathway with siRNA against CHOP. In addition, we investigated the temporal relationship between the expression of CHOP and p53 during chondrocyte apoptosis.

Materials and methods

Reagents. Sodium nitroprusside (SNP), a NO-donor, was purchased from Sigma (St. Louis, MO, USA). PBA was purchased from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nacalai Tesuque (Kyoto, Japan). Fetal bovine serum (FBS), collagenase type II, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, CA, USA).

Chondrocyte isolation and culture. The animal experiments in this study were designed according to the Guidelines for Animal Experimentation of Kumamoto University and were approved by the Animal Experiment Committee of Kumamoto University. Rat articular chondrocytes were isolated from slices of femoral head cartilage from 5-week-old Wistar rats (Japan SLC Inc., Hamamatsu, Japan) by a sequential enzyme digestion method using collagenase type II as described in a previous study (13). Isolated chondrocytes were plated in flasks at a density of $5x10^4$ cells/cm^2 in DMEM supplemented with 10% FBS, 100 U/ml streptomycin and 100 U/ml penicillin. The culture medium was replaced every 2 days. After 5 days in culture, the cells were detached using trypsin-EDTA and plated on culture plates at a density of $10x10^4$ cells/cm^2. Three days after passage, the cells were incubated in serum-free medium with or without PBA (3 mM) for 12 h and then treated with SNP (0, 0.5, 1 or 2 mM) for 24 h.

siRNA transfection. Stealth Select RNAi specific for *Chop* (RSS355093) and Stealth RNAi negative control Hi GC (12935-400) were purchased from Invitrogen. Rat chondrocytes were seeded at $5x10^4$ cells/cm^2 in antibiotic-free medium. After 24 h, the cells were transfected with 10 nM siRNA duplexes using lipofectamine RNAiMAX for 36 h (Invitrogen) according to the manufacturer's instructions. Following transfection, the cells were incubated in serum-free medium for 12 h and then treated with SNP (1 mM) for 24 h.

Nitrite/nitrate assay. Total NO was measured as its breakdown products, nitrite and nitrate, by the Griess reaction using a nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, culture medium from chondrocytes was reacted with nitrate reductase and its cofactor for 1 h at room temperature. After color development by the addition of Griess reagent, the amount of nitrate/nitrite was determined by absorbance at 540 nm.

Enzyme-linked immunosorbent assay (ELISA) for apoptosis. The extent of cultured apoptosis of chondrocytes was analyzed using Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. Following treatment, the cell lysate was incubated for 2 h at room temperature with anti-histone-biotin and anti-DNA-peroxidase antibodies. The absorbance of the samples was measured at 405 and 490 nm. For each experiment, the amount of protein in the cell lysate was assessed in separate wells, using the Quick Start Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA) to normalize the extent of cell apoptosis (11). For each experiment, the apoptosis enrichment factor was calculated as the absorbance (A405-A490 nm) of the cells treated with agents/absorbance (A405-A490 nm) of untreated control cells.

RNA extraction and real-time polymerase chain reaction (PCR). Total RNA was extracted from cultured cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) in combination with DNA digestion using DNase (Qiagen) and reverse-transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster, CA, USA). The procedures were performed according to the manufacturer's instructions. Quantitative real-time RT-PCR analysis was performed on an Applied Biosystems 7300/7500 Real-time PCR system using the TaqMan Gene Expression Master mix (Applied Biosystems) and TaqMan Gene Expression assays for *Chop* (Rn00492098-g1), *Grp78* (Rn01435771_g1), *p53* (Rn00755717_m1), and *Gapdh* (Rn99999916_s1) (Applied Biosystems). Reactions were carried out under the conditions: 2 min at 50˚C and 10 min at 95˚C; 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The relative quantification of the target gene to *Gapdh* was calculated using the ΔΔCt method (User Bulletin no. 2, Applied Biosystems).

Statistical analysis. Data were expressed relative to the mean value of cells treated without agents in each experiment. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) with the Scheffe's post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SNP induces apoptosis and ERS in chondrocytes. The generation of NO in SNP-treated chondrocytes was confirmed using the Griess reaction (Fig. 1A). Treating chondrocytes with SNP significantly increased apoptosis of chondrocytes at doses of ≥1 mM (Fig. 1B). *Chop* mRNA expression increased in SNP-treated chondrocytes in a dose-dependent manner (Fig. 1C). SNP also increased *Grp78* mRNA expression in chondrocytes (Fig. 1D), although this effect was reduced in a dose-dependent manner, and no significant difference was observed in the level of *Grp78* expression between 2 mM SNP-treated chondrocytes and the control cells (Fig. 1D). These results suggest that NO induces ERS in chondrocytes. On the basis of these results, 1 mM SNP was used in subsequent experiments.

ERS inhibitor PBA and Chop knockdown reduce SNP-induced apoptosis of chondrocytes. Although PBA had no significant effect on the generation of NO by SNP (Fig. 2A), PBA reduced

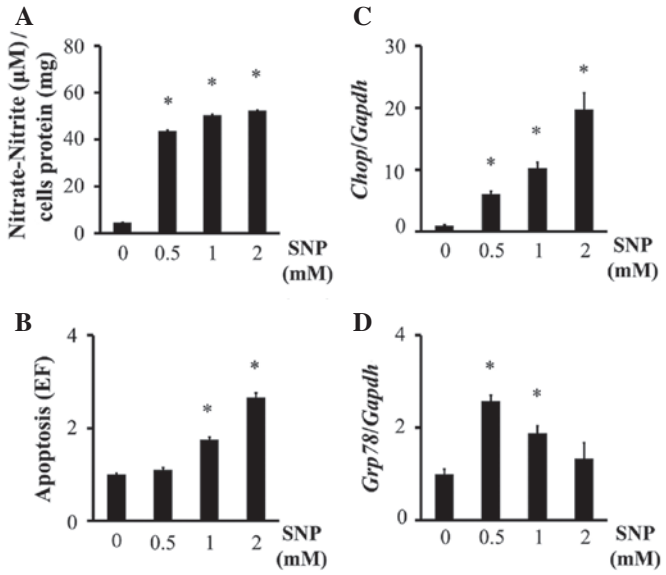

Figure 1. SNP increases ERS-associated mRNA expression and apoptosis in chondrocytes. Chondrocytes were treated with SNP (0-2 mM) for 24 h. (A) NO generation was measured using the Griess reaction. (B) Quantification of apoptosis was performed by ELISA. (C) *Chop* and (D) *Grp78* mRNA expression associated with ERS relative to *Gapdh* expression was measured by real-time PCR and normalized to samples without SNP. Values are represented as the mean ± standard error of the mean of three independent experiments performed in triplicate. *P<0.05 vs. samples without SNP. SNP, sodium nitroprusside; NO, Nitric oxide; ERS, endoplasmic reticulum stress.

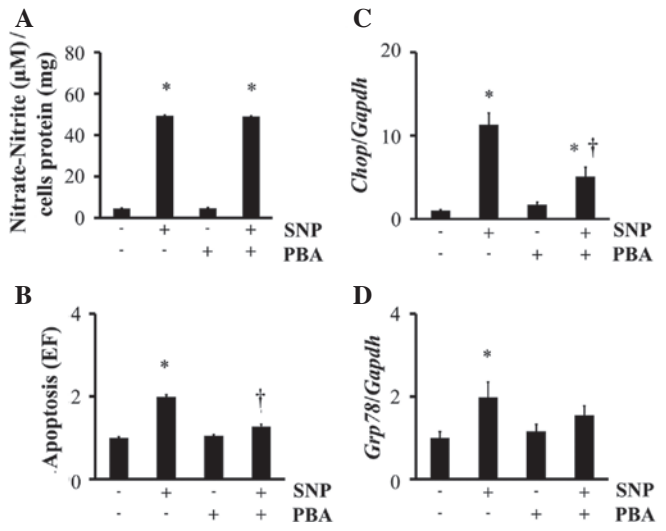

Figure 2. PBA suppresses ERS and apoptosis in chondrocytes. Chondrocytes were pretreated with PBA (3 mM) for 12 h and then stimulated by SNP (1 mM) for 24 h. (A) NO generation by SNP was measured using the Griess reaction. (B) Chondrocyte apoptosis was analyzed by ELISA. (C) *Chop* and (D) *Grp78* mRNA expression relative to that of *Gapdh* was measured by real-time PCR. The data were normalized to samples treated without agents. Values are presented as the mean ± ± standard error of the mean of three independent experiments performed in triplicate. *P<0.05 vs. samples without agents. †P<0.05 vs. samples with SNP alone. PBA, 4-phenylbutyrate; SNP, sodium nitroprusside; NO, Nitric oxide; ERS, endoplasmic reticulum stress.

apoptosis (Fig. 2B) and the mRNA expression of *Chop* (Fig. 2C) and *Grp78* (Fig. 2D) in SNP-treated chondrocytes.

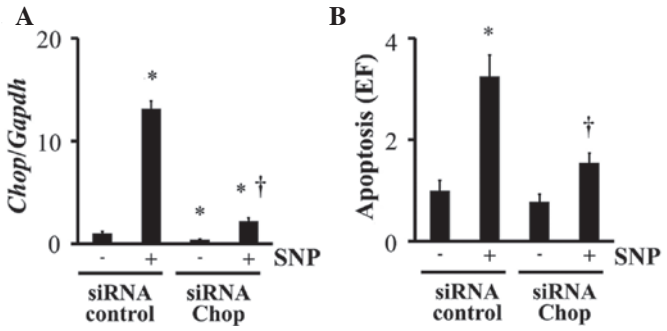

Figure 3. Inhibition of *Chop* expression reduces SNP-induced chondrocyte apoptosis. Chondrocytes were transfected with *Chop* or negative control siRNA and then stimulated with SNP (1 mM). (A) Twenty-four hours after stimulation, *Chop* mRNA expression relative to that of *Gapdh* was measured by real-time PCR in chondrocytes stimulated with SNP. (B) Chondrocyte apoptosis stimulated by SNP for 24 h was analyzed by ELISA. Data were normalized to samples treated without agents. Values are presented as the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate. *P<0.05 vs. samples without agents. †P<0.05 vs. samples treated with negative control siRNA and SNP. SNP, sodium nitroprusside.

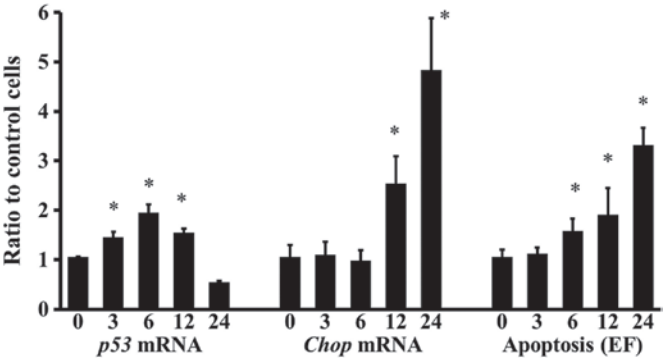

Figure 4. Expression of *Chop* and *p53* and changes in apoptosis in a time-dependent manner. Chondrocytes were treated with SNP (1 mM) for 24 h. The mRNA expression of *Chop* and *p53* was measured by real-time PCR. Chondrocyte apoptosis was analyzed by ELISA. Data were expressed relative to the mean value of control cells treated without SNP at each time point. Values of the graph are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. *P<0.05 vs. samples prior to treatment. SNP, sodium nitroprusside.

Chop knockdown was performed by siRNA transfection. *Chop* siRNA significantly inhibited *Chop* expression in chondrocytes, even when the cells were stimulated with SNP (Fig. 3A). siRNA treatment also reduced SNP-induced apoptosis of chondrocytes (Fig. 3B).

Temporal relationship between the expression of Chop and p53 and the induction of apoptosis. Three hours after chondrocytes were treated with SNP, *p53* expression significantly increased, but *Chop* expression and apoptosis were not enhanced (Fig. 4). *p53* expression of chondrocytes achieved a peak 6 h following treatment and was kept upregulated up to 12 h (Fig. 4). *Chop* expression increased in a time-dependent manner 12 h after SNP stimulation, whereas apoptosis of chondrocytes increased in a time-dependent manner 6 h after the stimulation (Fig. 4).

Discussion

In this study, we observed that SNP induced apoptosis of chondrocytes and SNP-stimulated chondrocytes showed an increase in the CHOP and GRP78 mRNA expression. These results are consistent with those of previous reports using *S*-nitroso-*N*-acetylpenicillamine (SNAP), another NO donor (12). Therefore, NO is an inducer of ERS in chondrocytes. In addition, we found that PBA treatment as well as the blockade of the CHOP expression was able to suppress NO-induced apoptosis of chondrocytes.

PBA has been reported to be a chemical chaperone that reduces the misfolding and mislocalization of mutant α1-antitrypsin (14) and the aggregation of Pael-R (15), and attenuates ERS in liver cells, mouse embryonic fibroblasts (16) and neuronal cells (15,17). In a preliminary experiment (data not shown), PBA was confirmed to also be useful as an ERS inhibitor in chondrocytes, based on the observation that PBA inhibited the expression of GRP78 and CHOP, as well as the tunicamycin-stimulated apoptosis in chondrocytes, a typical ERS inducer. Therefore, the suppressive effect of PBA on NO-stimulated apoptosis in chondrocytes determined that NO-induced ERS led chondrocytes to apoptosis. The results of the CHOP knockdown experiment also supported this conclusion.

In their study, Kim *et al* (4,5) demonstrated that p53 was responsible for NO-induced apoptosis of chondrocytes. In the present study, the p53 expression in NO-treated chondrocytes increased prior to the induction of apoptosis and decreased with sustained stimulation by NO, despite the increased apoptosis. By contrast, the upregulation of CHOP was induced following the induction of NO-stimulated apoptosis in chondrocytes. These results suggest that p53 plays a role in the apoptosis of chondrocytes mainly at the acute stage of NO stimulation, although this was not the case for ERS. However, similar to apoptosis, the CHOP expression was found to increase with sustained stimulation. Qu *et al* (18) demonstrated that ERS prevented p53 stabilization via glycogen synthase kinase-3β and inhibited p53-mediated apoptosis. Taking these findings into consideration, we assumed that ERS initially inhibited p53-mediated NO-induced apoptosis of chondrocytes, but as NO stimulation progressed, the persistent impairment altered the ERS responses of chondrocytes from protective to apoptotic. However, the mechanism of this switch remains unclear. Therefore, additional studies are required to clarify the role of ERS in NO-induced apoptosis.

The limitation of this study is that our observations are based on experiments using exogenous NO generated by SNP, a NO donor, rather than NO synthases-generated endogenous NO. However, endogenous NO may also induce ERS and lead to apoptosis in several cell types, such as pancreatic β cells (19) and macrophages (20). Therefore, endogenous NO-induced apoptosis of chondrocytes may also be mediated by ERS.

In conclusion, findings of the present study have demonstrated that ERS contributed to NO-induced apoptosis of chondrocytes, using pharmacological attenuation of ERS and blockade of the ERS-associated apoptotic pathway. However, the contribution of ERS appears to be limited to persistent impairment of NO stimulation. Previous studies have demonstrated that NO is the molecule most responsible for apoptosis of chondrocytes during cartilage degeneration (1-3). Therefore, our results support that ERS is involved in apoptosis of chondrocytes of degenerated cartilage. The importance of ERS in NO-induced apoptosis may provide insight into the pathology of cartilage degeneration. However, additional studies are required to investigate the role of ERS in cartilage biology.

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