Apoptotic Signaling Pathways in Mammalian Growth Plate Chondrocytes

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APOPTOTIC SIGNALING PATHWAYS IN MAMMALIAN

GROWTH PLATE CHONDROCYTES

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To my dear mother, Enling Li.

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SUMMARY

The growth plate resting zone consists of hyaline-like chondrocytes disbursed in a proteoglycan rich extracellular matrix. These cells give rise to the columns of the growth zone, consisting of progressively hypertrophic cells. Proliferation of resting zone chondrocytes induced by systemic and local stimuli is the driving force of longitudinal growth of long bones. Therefore, homeostasis of this cell population has great importance. Although the regulation of proliferation and differentiation of these cells has been well studied, little is known about the regulation of their apoptosis. We have previously shown that chelerythrine and tamoxifen induce apoptosis in resting zone chondrocytes in a nitric oxide (NO)-dependent pathway. In this study we explored two physiological apoptogens: inorganic phosphate (Pi) and 17β-estradiol (E_2). We found NO production is necessary in Pi-induced apoptosis. We also found that NO donors induced chondrocyte apoptosis by up-regulating p53 expression, Bax/Bcl-2 expression ratio and cytochrome C release from mitochondria, as well as caspase-3 activity, indicating that NO induces chondrocyte apoptosis in a mitochondrial pathway. Mitogen activated protein kinase (MAPK) activity was involved. A c-Jun N-terminal kinase (JNK) inhibitor, but not inhibitors of p38 or extracellular signal-regulated kinase (ERK1/2), was able to block NO-induced apoptosis, indicating that JNK is necessary in this pathway. Taken together, Pi elevates NO production, which leads to a mitochondrial apoptotic pathway dependent on JNK. On the other hand, although E_2 caused apoptosis in resting zone

chondrocytes in a dose-dependent manner, up-regulated p53 and Bax, and induced release of cytochrome C from the mitochondria, which indicated a mitochondrial apoptotic pathway, the apoptosis did not involve elevated nitric oxide production or MAPK as was found in Pi-induced apoptosis. This study elucidates the signaling pathway underlying Pi and E2-induced chondrocyte apoptosis. It has important implications on understanding the development of mammalian growth plate. It also provides further information about the physiological functions of estrogen on longitudinal bone growth.

Chapter 1

Introduction

1.1 Significance of the Proposed Research

Apoptosis or programmed cell death is a well-orchestrated process intrinsic to multiple-cellular eukaryotes that removes unwanted cells from the organism. Together with differentiation and proliferation, apoptosis contributes to the maintenance of homeostasis of the cell population, and its malfunction has grave consequences. In the growth plate, physiological chondrocyte apoptosis is well described (Ohyama et al., 1997; Hatori et al., 1995), and is associated with osteogenic markers (Roach et al., 1995a). Disruption of chondrocyte apoptosis in the hypertrophic zone of the growth plate results in the pathological elongation of growth plate in rickets (Donohue and Demay, 2002). Most research on growth plate chondrocyte apoptosis has been focused on the more mature prehypertrophic and hypertrophic cells (growth zone). Whereas little is known about the regulation of apoptosis in the less differentiated resting zone (reserve zone), we believe that research on this topic also has crucial importance for the following reasons. First, in epiphyseal fusion, the whole growth plate, including not only the growth zone but also the resting zone, is eliminated in an estrogen-dependent pathway (Weise et al.,

2001).   Second, during fracture healing, a cartilaginous callus is formed to carry out

endochondral ossification mimicking the same process in the growth plate (Bostrom,

1998; Vortkamp et al., 1998).   Agents regulating resting zone chondrocytes are likely

to act on early stages of this process for a rapid effect.   Last but not least, cartilage

tissue engineering is troubled by the low viability of chondrocytes in vitro, and

inhibition of resting chondrocyte apoptosis will certainly improve this situation.

In this study we will mainly focus on two apoptogens of resting zone

chondrocytes: inorganic phosphate (Pi) and 17β-estradiol (E$_2$).   The mechanisms of

apoptosis caused by these two agents will be examined in a rat costochondral growth

plate model (Boyan et al., 1988b).   Collectively, outcomes of this study will provide

us insights into the mechanism by which resting zone chondrocyte apoptosis is

regulated.   The information gained from this study will provide new knowledge

about regulation of endochondral bone formation, together with implications on the

development of new therapies on osteoarthritis, fracture healing and growth plate

disorders.

1.2 Animal Model

We used a rat costochondral growth plate model for the current study. This model has been used to study the regulation of proliferation, differentiation and matrix production of the growth plate chondrocytes by a broad range of stimuli. This model also allowed us to precisely separate the resting zone chondrocytes from the prehypertrophic and hypertrophic chondrocytes (the growth zone). Different factors regulate the two morphologically and biochemically distinct cell zones (Boyan et al., 1988b; Boyan et al., 1988a; Schwartz et al., 1988). For instance, resting zone chondrocytes respond to the vitamin D metabolite $24,25(OH)_2D_3$, whereas the growth zone chondrocytes respond to $1,25(OH)_2D_3$. Both vitamin D metabolites are produced actively in the growth plate in a regulated way by transforming growth factor $\beta1$ (TGF$\beta1$) in a zone-specific manner (Schwartz et al., 1992a). Both regulate the production of prostaglandin E2 (PGE2) (Schwartz et al., 1992b) and the level of interleukin-1 (IL-1) (Dean et al., 1997) in these cells. In addition, they also regulate protein kinase C (PKC) activity (Sylvia et al., 1993), but use different pathways: $1,25(OH)_2D_3$ dependent activation of PKC involves activation of phospholipase A2 (PLA$_2$) and arachidonic acid production (Boyan et al., 1998), whereas $24,25(OH)_2D_3$ dependent activation of PKC involves phospholipase D (PLD) activation (Sylvia et al., 2001a). Furthermore, the growth plate chondrocyte proliferation, differentiation and matrix production are also regulated by bone morphogenetic protein-2 (BMP2) in a zone-specific manner (Erickson et al., 1997). These studies demonstrate the value and significance of the rat costochondral growth plate model as a tool to investigate

the regulation of endochondral bone formation.

1.3 Review of Relevant Literature

Apoptosis is a process of programmed cell death occurring in metazoa (Kerr et al., 1972). Different from necrosis, another form of cell death, apoptotic cells retain its plasma membrane integrity and are later removed by macrophages. Apoptosis is distinguished by such typical morphological traits as shrinkage, blebbing and DNA fragmentation (Hengartner, 2000). A specific family of proteases, namely caspases, is responsible for carrying out apoptotic effects in cells (Thornberry and Lazebnik, 1998). Apoptosis is physiologically important because it provides the organism a mechanism to eliminate unwanted cells (Vaux and Korsmeyer, 1999). Malfunction of apoptosis may lead to cancer (Thompson, 1995). Conversely, excessive apoptosis can also lead to diseases such as Parkinson's, Alzheimer's or osteoarthritis (OA) (Thompson, 1995). There are two different apoptotic pathways. The intrinsic pathway involves mitochondria. Under apoptotic stimuli, change in mitochondrial membrane permeability caused by Bcl-2 family proteins induce cytochrome C release into the cytosol, which in turn induce caspase activation and apoptosis (Du et al., 2000). Alternatively, the extrinsic pathway involves a family of membrane receptors called death receptors (DR). DR, after binding to their ligands, form a complex called the death-inducing signaling complex (DISC), which activates caspases to carry out apoptosis (Walczak and Sprick, 2001).

Phosphate (Pi) is an anion required to make hydroxyapatite, the main inorganic component of calcified tissues, such as bones. Pi concentration of the cartilage

extracellular matrix is regulated locally by the action of $1,25(OH)_2D_3$-dependent

alkaline phosphatase, an enzyme that is enriched in extracellular matrix vesicles

(Anderson, 1995). Alkaline phosphatase activity increases towards the lower region

of the growth plate where cells hypertrophy, and when the ion product of Pi and

calcium concentration exceeds their dissociation constant, mineralization starts with

the formation of apatite crystals. Pi concentration of the cartilage extracellular matrix

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Nitric oxide (NO) is a small and uncharged compound that serves as a signaling

molecule in various organs and tissues of animals. It has significant physiological

roles in cardiovascular, neural and immune systems (Lowenstein and Snyder, 1992;

Bredt and Snyder, 1994; Mayer and Hemmens, 1997). In vivo, NO is mainly

synthesized by three NO synthases (NOSs): two constitutively expressed NOSs,

namely neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS), and an

inducible NO synthase (iNOS), which is activated by stimuli such as cytokines and

lipopolysaccharides (Stadler et al., 1991). NO mainly binds a soluble guanylate

cyclase (sGC), induces cyclic guanosine monophosphate (cGMP) production and

subsequently activates multiple cGMP-dependent protein kinases to achieve its

physiological functions (Denninger and Marletta, 1999). NO can also react with other

proteins to generate S-nitrosothiols, thereby eliciting a broader range of biological reactions, although not all of them are beneficial to the cell (Stamler and Hausladen, 1998; Stamler, 1994).

In cartilage tissues, NO has significant roles in regulating many chondrocyte functions. In articular cartilage, NO inhibits integrin outside-in signaling pathways (Clancy et al., 1997), and induces breakdown of extracellular matrix (Amin and Abramson, 1998) as well as apoptosis (Blanco et al., 1995). NO-induced chondrocyte apoptosis is responsible for the pathological cell loss in osteoarthritis (van den Berg, 2001). In chick growth plate chondrocytes, NO is required for physiological chondrocyte apoptosis (Teixeira et al., 2001). Besides regulating chondrocyte apoptosis, NO production is also important in maturation of growth plate chondrocytes (Teixeira et al., 2005). Therefore, NO serves as an extremely important regulator in both physiological and pathological forms of chondrocyte apoptosis.

Estrogen is a major female sex hormone, secreted in the ovaries in females or converted from testosterone by aromatases in both sexes (MacGillivray et al., 1998). It is a key regulator of growth plate development as demonstrated by its ability to induce pubertal growth spurts and epiphyseal fusion (Grumbach, 2000; Rodd et al., 2004). Humans with mutations in either estrogen receptor alpha (ERα) or aromatase have delayed epiphyseal fusion, resulting in taller stature (Smith et al., 1994; MacGillivray et al., 1998). Estrogen has been used to treat young females with excessive height (Svan et al., 1991), whereas aromatase inhibitors have been used to

improve height in young male (Hero et al., 2005).   Although part of estrogen's effects on bone growth are through stimulation of the growth hormone (GH)-insulin like growth factor-1 (IGF-I) axis, estrogen receptors are expressed in both human and rodent growth plates (Egerbacher et al., 2002; van der Eerden et al., 2002a) as well as several enzymes involved in estrogen metabolism (van der Eerden et al., 2002b), implying a direct action of estrogen on the growth plate. This has been confirmed in both human and rodent research (Blanchard et al., 1991; Sylvia et al., 1998).

Estrogen induces epiphyseal fusion by exhausting the proliferation potential of growth plate chondrocytes (Weise et al., 2001).   High doses of estrogen also induce activation of caspase-3 in the growth plate, indicating involvement of apoptosis (Takano et al., 2008).   The effect of estrogen on growth plate fusion is similar in both sexes (Rodd et al., 2004), which is directly in contrast to its female-only effects on proliferation and differentiation of both rat growth plate chondrocytes and human articular chondrocytes (Kinney et al., 2005; Sylvia et al., 1998).

Estrogen exerts its physiological functions on cells via the classical nuclear receptor pathway.   Moreover, estrogen also acts through rapid and nongenomic pathways that do not require new protein synthesis in reproductive tissues (Revelli et al., 1998), brain (Toran-Allerand et al., 1999) and bone (Oursler, 1998), in part through membrane-associated estrogen receptors.   The effects of estrogen on proliferation and differentiation of rat costochondral resting zone chondrocytes involve a membrane receptor-related pathway (Sylvia et al., 1998; Sylvia et al., 2001b), which acts through activation of protein kinase C (PKC) (Sylvia et al., 1994) and mitogen activated protein

kinase (MAPK) (McMillan et al., 2006).   Whether chondrocyte apoptosis is directly

induced by estrogen and whether this process involves membrane receptors is not clear.

1.4 Specific Aims and Experimental Design

The ***overall goal*** of this thesis was to determine the phosphate and estrogen apoptotic signaling pathway in resting zone cells and ways to regulate it. The ***central hypothesis*** was that apoptosis in the resting zone of mammalian growth plate is induced by phosphate and estrogen involving nitric oxide production, MAP kinases and a mitochondrial apoptotic pathway. The problem was addressed by three specific aims.

Aim 1: To determine the role of nitric oxide production in regulation of phosphate-induced apoptosis in resting zone chondrocytes

The ***objective*** of this aim was to determine the role of NO production in regulating resting zone chondrocyte apoptosis. The ***working hypothesis*** was that blocking NO production would be able to block resting zone chondrocyte apoptosis induced by Pi. To address this problem, we first established Pi as an effective apoptogen in the rat growth plate chondrocyte model. The effects of NO on chondrocyte viability and apoptosis were subsequently assessed in chondrocytes treated with Pi. Furthermore, the effects of NO synthase inhibitors and NO synthase knockout on Pi-induced apoptosis were assessed.

Aim 2: To determine the pathway that leads to NO-induced chondrocyte apoptosis

The *objective* of this study was to investigate the signaling pathway induced by NO to carry out the apoptotic function. The *working hypothesis* was that NO would induce resting zone chondrocyte apoptosis via a mitochondrial pathway that involves MAP kinases. To test the hypothesis, we first established NO donors as effective apoptogens. Subsequently, the effects of NO donors on mitochondrial apoptosis pathway were evaluated. We also investigated whether blocking MAP kinases were able to block NO donor-induced chondrocyte apoptosis.

Aim 3:   To determine whether estrogen induces chondrocyte apoptosis via an NO mechanism

The *objective* of this study was to elucidate the mechanism of estrogen-induced apoptosis in growth plate chondrocytes. The *working hypothesis* was that estrogen would induce chondrocyte apoptosis in a sex-independent manner, involving NO and mitochondrial pathway, similar to Pi-induced apoptosis pathway. To test the hypothesis, we first established E_2 as an effective apoptogen. Subsequently, the effects of E_2 on chondrocyte of both sexes and different maturation states were assessed. The involvement of NO, MAP kinases and mitochondrial apoptosis pathway in E_2-induced apoptosis were also assessed. We also assessed the roles of different estrogen receptors in E_2-induced apoptosis.

Chapter 2

Material and Methods

2.1 Material

Thrombin peptide TP508 was a gift from OrthoLogic (Tempe, AZ). E_2, E_2-BSA, sodium phosphate monobasic, N_ω-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-18), S-nitrosoglutathione (SNOG), sodium phosphonoformate tribasic (PFA), ICI182780, 2,3-diaminonaphthalene (DAN), SB203580, PD98059, sodium citrate, paraformaldehyde and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO). JNK inhibitor II was purchased from CalBiochem (San Diego, CA). Caspase-1 inhibitor Z-YVAD-FMK and caspase-3 inhibitor Z-DEVD-FMK were purchased from BioVision (Mountain View, CA). Anti-ERα36 was purchased from Chi Scientific (Maynard, MA). 3,3′-diaminodbenzidine (DAB) substrate was purchased from Roche Applied Science (Indianapolis, IN). Cytochrome C antibodies were purchased from BD Biosciences (San Jose, CA) and BioVision (Mountain View, CA).

2.2 Chondrocyte Cultures

We used a rat costochondral growth plate model for the current study. This model has been used to study the regulation of proliferation, differentiation and matrix production of the growth plate chondrocytes by a broad range of stimulus. This model also allows us to precisely separate the resting zone chondrocytes from the prehypertrophic and hypertrophic chondrocytes (the growth zone). Different factors regulate the two morphologically and biochemically distinct cell zones (Boyan et al., 1988b; Boyan et al., 1988a; Schwartz et al., 1988). Resting zone chondrocytes respond to the vitamin D metabolite $24R,25(OH)_2D_3$, whereas the growth zone chondrocytes respond to $1\alpha,25(OH)_2D_3$. Both metabolites regulate protein kinase C (PKC) activity (Sylvia et al., 1993), but through different pathways (Boyan et al., 1998; Sylvia et al., 2001a; Schwartz et al., 2003; Schwartz et al., 2005). These studies demonstrate the value and significance of the rat costochondral growth plate model as a tool to investigate the regulation of endochondral bone formation.

Unless otherwise indicated, chondrocytes were isolated from the resting zone of the costochondral cartilage of 100-125g Sprague-Dawley rats (Harlan, Indianapolis, IN) as described previously (Boyan et al., 1988b). Initial experiments compared cells from male and female donors to rule out any sex-specific differences in mechanism. In some experiments, resting zone chondrocytes were also isolated from 12-week old male wild-type (C57BL/6) (Harlan, Indianapolis, IN) or eNOS-/- mice (C57BL/6 background) (The Jackson Laboratory, Bay Harbor, ME) (Shesely et al., 1996). At third passage confluence, the chondrocytes were subpassaged into plates or flasks with six separate cultures per variable per experiment.

2.3 DNA Fragmentation Assays

DNA fragmentation was measured by two different assays. The first assay was conducted using the Cell Death Detection ELISA+ kit (Roche) following manufacturer's instructions with a few modifications. Briefly, after treatment with E2 for 24 hours, cells were harvested by trypsinization and counted using a Beckman-Coulter Z1 Cell Counter (Beckman Coulter, Fullerton, CA). Cells were lysed using the provided lysis buffer and 20μl cell lysate were mixed with the provided immunoreagent containing two antibodies for the sandwich reaction of ELISA in a plate pre-coated with streptavidin and incubated for 2 hours. ABTS (provided in the kit), a substrate of the antibody-linked horseradish peroxidase, was added to the wells and color allowed to develop. Absorbance was measured at 405 nm for each well and normalized with cell number to determine the extent of apoptotic cell death.

The second assay was adapted from Grey et al. (Grey et al., 2002) with modifications. Cells were seeded in 24-well plates and grown to near confluence. The cells were labeled with ^{3}H-thymidine (0.5μCi/well) for 4 hours. The culture media, together with unincorporated ^{3}H-thymidine were discarded. After washing with cold DMEM twice, the cells were treated with effectors for 24 hours. The cells were harvested with trypsin-EDTA and collected. Then the cells were lysed with TE buffer (10mM Tris-HCl; 1mM EDTA, pH 7.4; 0.2% Triton X-100). To ensure the lysis of the cells, the samples were frozen and thawed three times. The lysates were subsequently centrifuged at 13,000g for 15 minutes. The radioactivity of both the supernatants and the pellets were measured using a scintillation counter. The

percentage of fragmented DNA was calculated by dividing the radioactivity of the supernatants (representing the fragmented DNA) by the sum of the radioactivity of the supernatants and the pellets (representing the total DNA).

2.4 TUNEL

TUNEL staining was conducted using an In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) in order to verify that DNA fragmentation had occurred (Kasagi et al., 1994). Briefly, cells were treated with E_2 for 24 hours. Media were discarded and the cells were air dried and fixed for 1 hour in 4% paraformaldehyde in PBS (pH=7.4). The fixed cells were rinsed with PBS and blocked with 3% hydrogen peroxide in methanol for 10 minutes. Next, cells were rinsed with PBS and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 30 minutes. The cells were then rinsed with PBS and incubated with TUNEL reaction mixture for 1 hour. TUNEL reaction mixture was made by mixing label solution and enzyme solution from the kit. Cells were rinsed and incubated with converter-POD (provided by the kit) for 30 minutes, and then incubated with POD substrate for 10 minutes. Apoptotic cells were observed using a light microscope.

2.5 p53 Assay

The amount of p53 protein was determined by p53 pan ELISA kit (Roche Applied Science, Indianapolis, IN). Briefly, after treatment with E_2 for 24 hours, cells were lysed. 100µl lysate were mixed with the same amount of anti-p53-peroxidase (POD) in each well of a microplate pre-coated with both streptavidin and

anti-p53-biotin. After two hours incubation, the supernatants were discarded and the wells were rinsed. 200µl substrate buffer (provided in the kit) was added to the well and color developed for 20 minutes. Absorbance was measured at 450nm. The p53 abundance was determined by fitting the absorbance into a standard curve with known concentrations of human p53. All results were normalized to total protein concentration.

2.6 Western Blots

Effects of E_2, SNOG and Pi on Bax/Bcl-2 expression were determined by Western blot of cell culture lysates prepared from female rat chondrocytes. Samples (50µg) were resolved on 10% SDS-polyacrylamide gels. Blots of the gels were probed with a mouse monoclonal antibody to Bax (sc-7480, Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal antibody to Bcl-2 (#2876, Cell Signaling Technology, Danvers, MA). Immunoreactive bands were detected using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized using enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ). Controls were performed with an anti-GAPDH antibody (Mab327, Chemicon International, Temecula, CA). Immunoreactive bands were scanned and their relative intensities were determined using a VersaDoc imaging system (Bio-Rad, Hercules, CA).

Effects of Pi on eNOS, iNOS and nNOS expression were determined by

Western blot as described above with rabbit polyclonal antibodies to eNOS (ab5589, Abcam, Cambridge, MA) and nNOS (ab63602, Abcam, Cambridge, MA), and a mouse monoclonal antibody to iNOS (ab49999, Abcam, Cambridge, MA). Controls were performed with an anti-GAPDH antibody (Mab327, Chemicon International, Temecula, CA).

2.7 Macro Protein Assay

All samples were normalized to total protein content using the Pierce Macro BCA Protein Assay Reagent kit from Pierce Biotechnology (Rockford, IL). Reagents A and B were mixed in a 50:1 ratio to make the working reagent. 5 μl of each sample was aliquoted in duplicate to 96-well plates, and 200μl of the working reagent was added to the sample plates. The plate was incubated at 37°C for 30 min and read in the Bio-Rad microplate reader at 570 nm. The protein concentration of each sample was determined by fitting the absorbance into a standard curve with known concentrations of bovine serum albumin (BSA).

2.8 Cell Viability Assay

Cell viability was determined using an MTT assay kit (Promega, Madison, WI). Briefly, after treatment with E_2 for 24 hours, media were discarded and replaced with fresh media with 15% MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated for 4 hours to allow color development. Absorbance was measured at 570nm with reference wavelength at 655nm. Results were presented as

absorbance of treatment groups divided by the control group.

2.9 Nitric Oxide Production

NO production was evaluated by determining the concentration of accumulated nitrite in the culture media using a fluorescence assay with 2,3-diaminonaphthalene (DAN) as originally described by Misko et al. (Misko et al., 1993) with modifications. Briefly, after the cells were treated for 24 hours, media were preserved by freezing and the cells were lysed to determine the protein concentration by macro protein assay. NO was indirectly measured as nitrite/nitrate (NO_x) by incubating the media with DAN in a strong acidic environment (2.5mg DAN in 50ml 0.62M HCl). The reaction was terminated with 2.8M NaOH. Fluorescence was measured with excitation at 364nm and emission at 406nm. NO_x was determined by fitting the fluorescence with a standard curve with known concentrations of nitrite.

2.10 Caspase-3 Assays

Caspase-3 activity was assayed using a Caspase 3 Assay Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instruction. Briefly, after treatment with apoptogens for 24 hours, cells were lysed. Acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin, a substrate of caspase-3, was added to the cell lysates. After one hour incubation, fluorescence was measured at excitation and emission wavelength of 360 and 460nm respectively. Caspase-3 activity was calibrated using a pNA substrate standard curve and normalized to protein concentrations.

Alternatively, Caspase-3 activity was assayed using the Caspase-3 Colorimetric Assay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, chondrocytes were grown in 6-well plates with reagents. After 24 hours, media were discarded. Cells were lysed using lysis buffer provided in the kit in 4°C for 10 minutes. 50μl cell lysates were mixed with same amount of reaction buffer and 5μl DEVD-pNA, and incubated at 37°C for 2 hours. Absorbance was measured at 405nm. Results were normalized to total protein concentration.

2.11 Cytochrome C Release

To measure cytochrome C release, we first separated cytosol lysate and mitochondrial extract using Cytochrome C Releasing Apoptosis Assay Kit (BioVision, Mountain View, CA). Briefly, after treatment with apoptogens for 24 hours, cells were lysed by homogenization in the cytosol extraction solution provided by the kit. Cell debris was removed by centrifugation at 700g for 10 minutes. Cytosolic and mitochondrial fractions were separated by centrifugation at 10000g for 30 minutes. Cytochrome C abundance in both fractions was measured by Western blot analysis as described above using a mouse monoclonal antibody provided by the kit. Moreover, cytosolic controls were performed with an anti-GAPDH antibody (Mab327, Chemicon International, Temecula, CA), and mitochondrial controls were performed with an anti-cytochrome C Oxidase IV (ab14744, Abcam, Cambridge, MA).

2.12 Statistical Analysis

All cell culture experiments were repeated at least twice. For each experiment, there were six independent cultures for each variable, and the results are shown as the mean ± standard error of at least five individual cultures. Statistical significance was determined using Wilcoxon signed-rank tests for all experiments whose results were presented as treatment/control or ANOVA and post-hoc testing with Bonferroni's modification of Student's t-test for all other experiments. The threshold of significance was set as P-value <0.05.

Chapter 3

The Role of NO in Pi-induced Apoptosis

3.1 Hypothesis and Rationale

The working *hypothesis* of Aim 1 was that blocking NO production would be able to block resting zone chondrocyte apoptosis induced by Pi. The *rationale* of this study was based on the following observations. Related studies showed that Pi induced apoptosis by elevating NO production in avian fetal chondrocytes (Teixeira et al., 2001). We had previously found that chelerythrine and tamoxifen-induced resting zone chondrocyte apoptosis was rescued by TP508, which blocked NO production (Zhong et al., 2008). Therefore, it is probable that Pi also induce resting zone chondrocyte apoptosis by elevating NO production.

3.2 Pi Induced Resting Zone Chondrocyte Apoptosis

Pi induced apoptosis in a dose-dependent manner. Chondrocyte cell viability was reduced, with 7.5mM Pi causing a 28% drop in MTT activity compared with control (Figure 1A). DNA fragmentation was increased, based on a significant increase in TUNEL staining (Figure 1B) in cultures treated with 7.5mM Pi and a 4.4-fold increase in [^{3}H]-thymidine-labeled DNA fragments compared with control (Figure 1C). The effect of Pi required regulated uptake via the NaPi transporter (Figure 1D). Treatment with PFA also blocked the effect of Pi on apoptosis. PFA treatment restored the effect caused by Pi treatment to control levels. The effects of Pi were not sex-dependent: the response of male cells to Pi was similar to the response of female cells (Table 1).

Pi regulated p53 abundance in the resting zone chondrocytes. Although 2.5mM Pi did not significantly increase p53, 5mM and 7.5mM Pi treatment increased p53 protein 2.6 and 2.8-fold, respectively (Figure 2A). Pi also induced a dose-dependent increase in levels of Bax and a dose-dependent decrease in levels of Bcl-2 (Fig 2B). The Bax/Bcl-2 ratio peaked at 7.5mM Pi treatment, with a 6-fold increase compared with control.

These results indicated that Pi did increase resting zone chondrocyte apoptosis in a sex-independent manner.
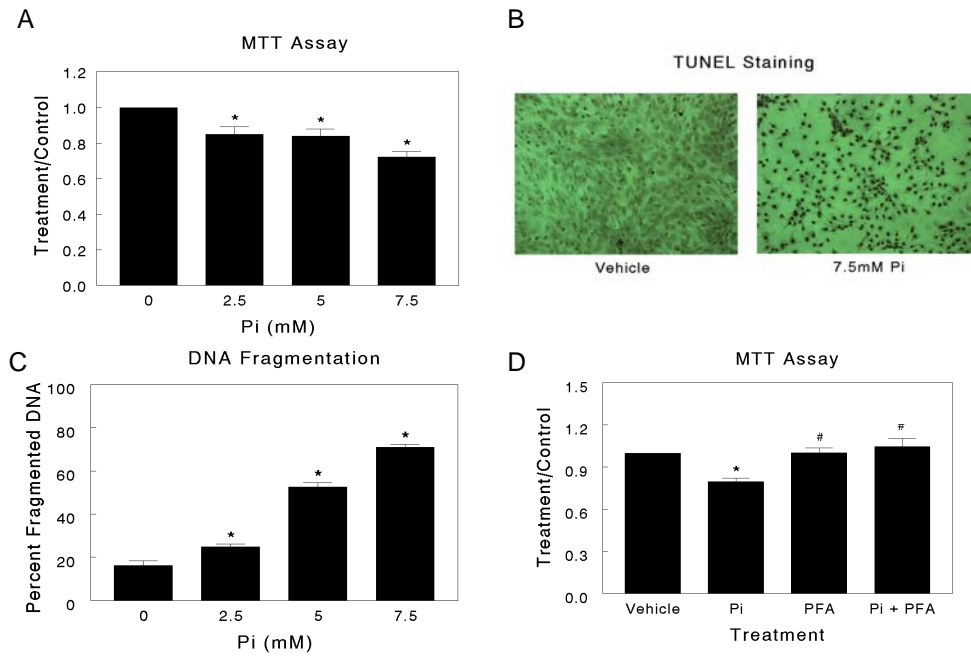
Figure 1 Pi-induced chondrocyte apoptosis. A,B,C: Cells were treated 0, 2.5, 5 or 7.5mM Pi (A, C) or just 0 and 7.5mM Pi (B) for 24 hours. MTT assay (A), TUNEL staining (B) and DNA fragmentation (C) were measured. D: Cells were treated with 1mM PFA for 24 hours, with half of the cultures also receiving 7.5mM Pi as apoptogen. Cell viability was measured by MTT assay. Data are presented as mean ± SEM (n=6) (A,C,D). *: $p<0.05$ vs. control; #: $p<0.05$ vs. Pi group.

Effect of Pi on Apoptosis of Male RC Cell Apoptosis

Phosphate (mM)	MTT (T/C)
0	1.00
2.5	0.87± 0.03*
5	0.83± 0.03*
7.5	0.81± 0.08*

Table 1 Effect of Pi on male RC chondrocyte cell viability.   RC male cells were treated with 0, 2.5, 5 or 7.5mM Pi for 24 hours.   Cell viability was measured by MTT assay.   *: $p<0.05$ vs. control.
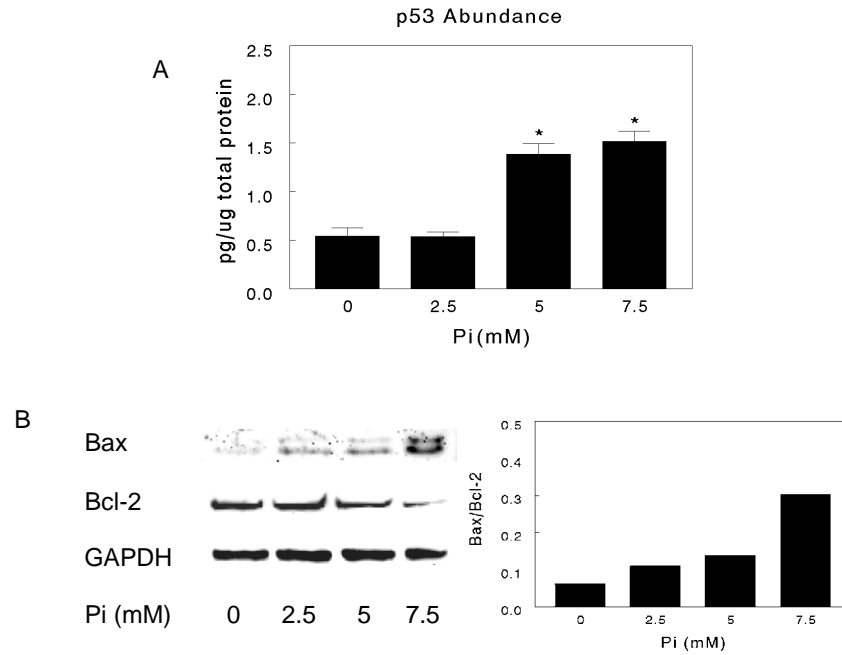
Figure 2 Effect of Pi on expression of p53 (A), Bax and Bcl-2 (B). Cells were treated with 0, 2.5, 5 or 7.5mM Pi for 24 hours. Expression of p53 was measured using the pan p53 ELISA kit. Expression of Bax and Bcl-2 was measured by western blot, with GAPDH as control. Data presented are means ± SEM (n=6) (A) or from one representative experiment (B). *: p<0.05 vs. control (A).

3.3 TP508 Blocked Pi-induced Chondrocyte Apoptosis by Blocking NO

Production

Pi induced resting zone chondrocyte NO production in a dose-dependent manner. Although 2.5mM Pi did not significantly increase NO production, 5mM Pi and 7.5mM Pi increased NO production 2.9-fold and 3.8-fold, respectively (Figure 3A). TP508 was able to block NO production caused by 7.5mM Pi. 7.5mM Pi significantly increased NO production, and 7μg/ml TP508 restored NO production to the base line level (Figure 3B). A similar trend was observed in Pi-induced apoptosis. 7.5mM Pi increased DNA fragmentation 2.8-fold compared with control, and 7μg/ml TP508 restored DNA fragmentation almost to the control level (Figure 3C). Both L-NMMA, a universal NO synthase inhibitor, and L-NAME, an iNOS and eNOS inhibitor was able to block Pi-induced apoptosis (Figure 4) in a pattern comparable to TP508. These results indicated that blocking Pi-induced NO production correlated with the inhibition of Pi-induced apoptosis.
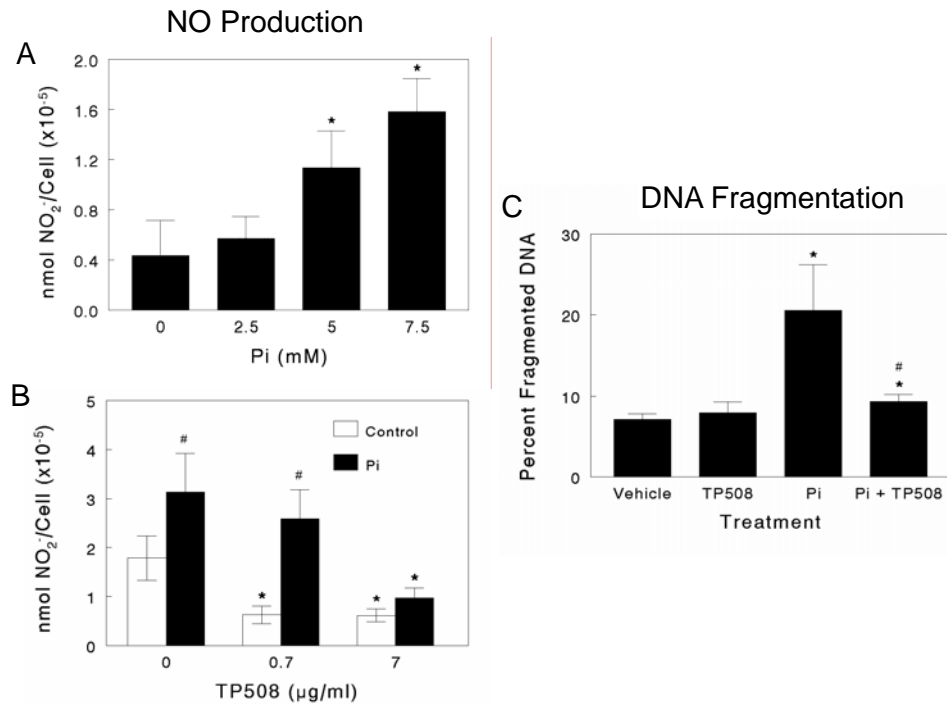
Figure 3 Inhibition of Pi-induced chondrocyte apoptosis and NO production by TP508.   A: cells were treated with 0, 2.5, 5 or 7.5mM Pi for 24 hours.   NO production was measured by DAN assay; B: cells were treated with 0, 0.7 or 7μg/ml TP508 for 24 hours, half of the cultures also receiving 7.5mM Pi.   NO production was measured by DAN assay; C: cells were treated with 0 or 7μg/ml TP508 for 24 hours, half of the cultures also receiving 7.5mM Pi.   Apoptosis was measured by DNA fragmentation assay.   Data are presented as mean ± SEM (n=6).   *: $p < 0.05$ vs. control; #: $p < 0.05$ vs. non-Pi groups (B) or non-TP508 groups (C).
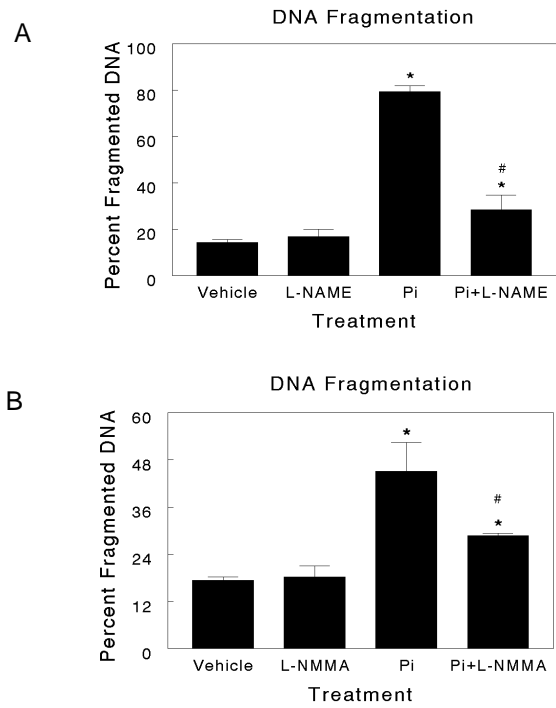
Figure 4 Effect of NO synthase inhibitors on Pi-induced chondrocyte apoptosis. Cells were treated with 0 or 7.5mM Pi for 24 hours. Half of the cultures also received NO synthase inhibitor 50μM L-NAME (A) or 1mM L-NMMA (B). Apoptosis was measured by DNA fragmentation assay. Data are presented as mean ± SEM (n=6). *: $p < 0.05$ vs. control; #: $p < 0.05$ vs. non-L-NAME groups (A) or non-L-NMMA groups (B).

3.4 Pi-induced Chondrocyte Apoptosis was through eNOS

Moreover, Pi increased eNOS protein abundance in a dose-dependent manner (Figure 5). 7.5mM Pi induced a 2.7-fold increase in eNOS protein abundance compared with control, although lower concentrations of Pi did not cause this change (Figure 5). 2.5mM Pi actually reduced eNOS expression compared with control. No iNOS or nNOS expression was detected, with or without Pi treatment.

Although Pi increased apoptosis in wild-type mouse chondrocytes (Figure 5A,B), Pi had little (Figure 5D) or no (Figure 5E) effect on apoptosis in eNOS-/- chondrocytes as judged by both DNA fragmentation assay and MTT assay, respectively. Pi also did not cause any change in NO production in eNOS-/- chondrocytes (Figure 5F), in stark contrast to its stimulatory effect in wild-type chondrocytes (Figure 5C). These results indicated that eNOS expression was key to Pi-induced chondrocyte apoptosis.

A

eNOS

GAPDH

Pi (mM)   0   2.5   5   7.5

B
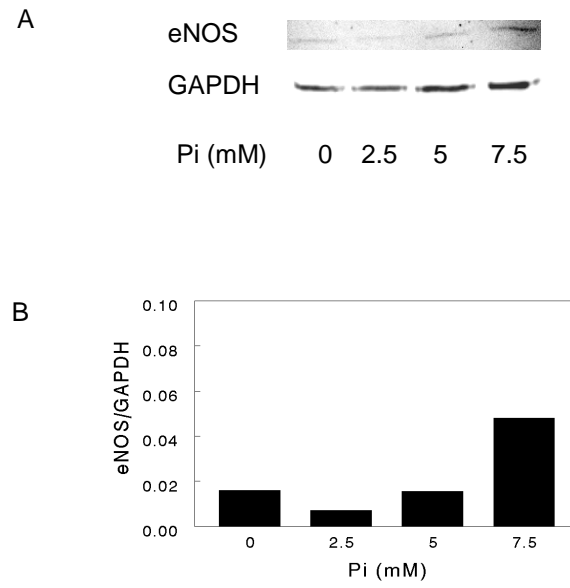
Figure 5 Pi-induced eNOS expression.   Cells were treated with 0, 2.5, 5 or 7.5mM Pi for 24 hours. eNOS expression was measured by Western Blot and normalized to GAPDH.
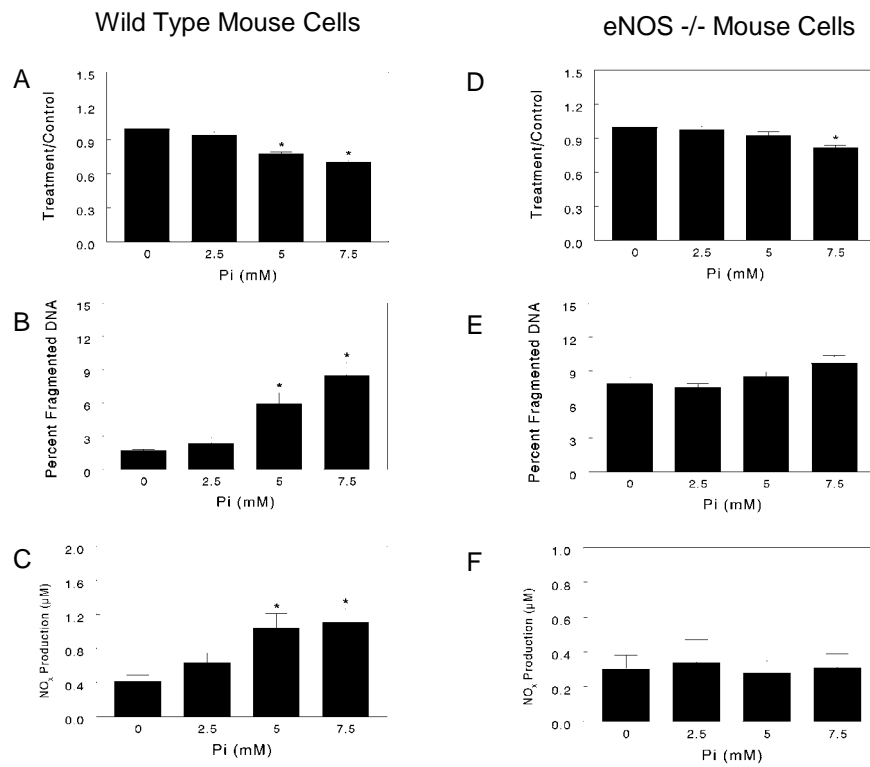
Figure 6 Comparison of the effects of Pi on wild-type and eNOS-/- mouse chondrocytes. Resting zone chondrocytes isolated from wild-type (A,B,C) and eNOS-/- mice mice (D,E,F) were treated with 0, 2.5,5 or 7.5mM Pi for 24 hours. Cell viability (A,D), DNA fragmentation (B,E) and NO production (C,F) were measured. Data are presented as mean $\pm$ SEM (n=6). *: p<0.05 vs. control.

3.5 Conclusion

The results above indicate that Pi dose-dependently increases chondrocyte apoptosis. This process requires NO production. Blocking NO production by NO synthase inhibitors or knocking out eNOS is able to inhibit the apoptotic effect of Pi.

Chapter 4

Signaling Pathway of NO-induced Chondrocyte Apoptosis

4.1 Hypothesis and Rationale

The working *hypothesis* of this aim was that NO induces resting zone chondrocyte apoptosis via a mitochondrial pathway that involves MAP kinases. The *rationale* was that elevated NO production is related to both physiological and pathological apoptosis of chondrocytes (van den Berg, 2001; Teixeira et al., 2001). The NO donor SNOG induces fetal chicken chondrocyte apoptosis in a mitochondrial pathway (Teixeira et al., 2001). MAP kinases JNK and p38 have been associated with apoptosis induced by multiple stress factors in a wide range of cells (Strniskova et al., 2002). Therefore, it is probable that NO induces resting zone chondrocyte apoptosis through a mitochondrial pathway involving either JNK or p38.

4.2 NO Donors Induced Chondrocyte Apoptosis

To investigate the direct effects of NO on resting zone chondrocytes, we employed two NO donors, NOC-18 and SNOG. Both decompose in media to slowly generate free NO molecules. Experiments using NO donors confirmed that NO induced apoptosis in the resting zone chondrocyte cultures. Treatment with both NOC-18 and SNOG resulted in a dose-dependent NO release into the media (Figure 7A) and both NO donors induced chondrocyte apoptosis in a dose-dependent manner as judged by MTT activity (Figure 7B) and DNA fragmentation (Figure 7C). The effects of NO were not sex-specific. SNOG caused a similar increase in NO release and apoptosis in male chondrocytes as was noted in female cells (Table 2,3). The effect of SNOG on chondrocytes was due to released NO. When SNOG was decomposed by UV light before adding to the media, it lost its apoptotic effect (Table 4).
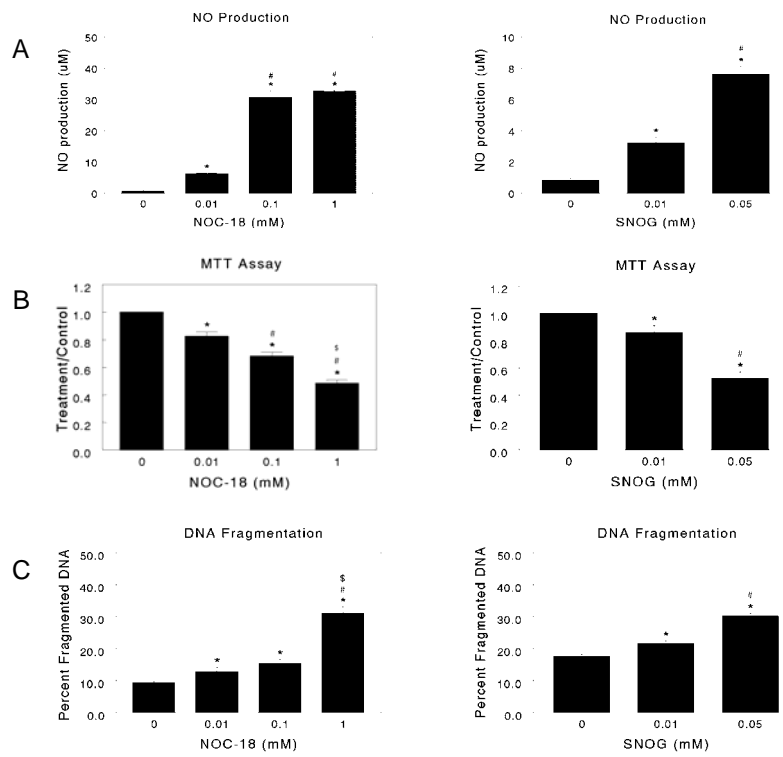
Figure 7 Effect of NO donors on chondrocyte apoptosis.   Cells were treated with 0, 0.01, 0.1 or 1mM NOC-18 or 0, 0.01 or 0.05mM SNOG for 24 hours.   NO production was measured by DAN assay (A). Cell viability was measured by MTT assay (B).   Apoptosis was measured by DNA fragmentation assay (C).   Data are presented as mean ± SEM (n=6).   *: p<0.05 vs. control; #: p<0.05 vs. 0.01mM NOC-18 or SNOG groups; $: p<0.05 vs. 0.1mM NOC-18 groups.

Effect of SNOG on NO Production of Male RC Cells

SNOG (mM)	NO Production (μ M)
0	1.5± 0.1
0.05	8.1± 0.4*
0.5	39.6± 0.4*#

Table 2 Effect of SNOG on NO production of male resting zone chondrocytes.   Cells were treated with 0, 0.05 or 0.5mM SNOG for 24 hours.   NO production was measured by DAN assay.   Data are presented as mean ± SEM (n=6).   *: p<0.05 vs. control; #: p<0.05 vs. 0.05mM SNOG group.

Effect of SNOG on Cell viability and Apoptosis of Male RC Cells

SNOG (mM)	DNA Fragmentation	MTT
0	15.53± 0.64	1.00
0.01	18.09± 0.72*	0.68± 0.06*
0.05	24.00± 4.42*	0.59± 0.04*

Table 3 Effect of SNOG on cell viability and apoptosis of male resting zone chondrocytes.   Cells were treated with 0, 0.01 or 0.05mM SNOG for 24 hours.   Cell viability was measured by MTT assay, and the results were presented as treatment vs. control.   Apoptosis was measured by DNA fragmentation assay, and the results were presented as percentage fragmented DNA.   Data are presented as mean ± SEM (n=6).   *: $p < 0.05$ vs. control.

Effect of Fresh and Decomposed SNOG on Cell Viability of Female RC Cells

SNOG (mM)	Fresh	Decomposed
0	1.00	
0.01	0.90± 0.03*	0.92± 0.03
0.05	0.75± 0.03*	0.97± 0.02#
0.5	0.66± 0.01*	0.90± 0.03*#

Table 4 Effect of fresh and decomposed SNOG on cell viability of female resting zone chondrocytes. Cells were treated with 0, 0.01, 0.05 or 0.5mM SNOG. Half of the cultures received SNOG decomposed before use. Cell viability was measured by MTT assay and the results were presented as treatment vs. control. Data are presented as mean ± SEM (n=6). *: $p<0.05$ vs. control; #: $p<0.05$ vs. fresh SNOG groups.

4.3 NO Induced Chondrocyte Apoptosis through a Mitochondrial Pathway

NO acted via a mitochondrial pathway involving increased p53 abundance and caspase activity. Compared with control culture, 0.05mM SNOG induced a 2.3-fold increase in cytosolic/mitochondria cytochrome C ratio (Figure 8A). SNOG also dose-dependently increased caspase-3 activity. 0.05mM SNOG induced a 1.6-fold increase in caspase-3 activity (Figure 8B). Similarly, 0.05mM SNOG increased p53 protein abundance 1.6 fold compared with control (Figure 8C). SNOG induced a dose-dependent increase in Bax/Bcl-2 ratio. SNOG caused a small but significant increase in Bax expression, but had no effect on Bcl-2 expression, resulting in a 1.5-fold increase in Bax/Bcl-2 in culture treated with 0.5mM SNOG (Figure 9).
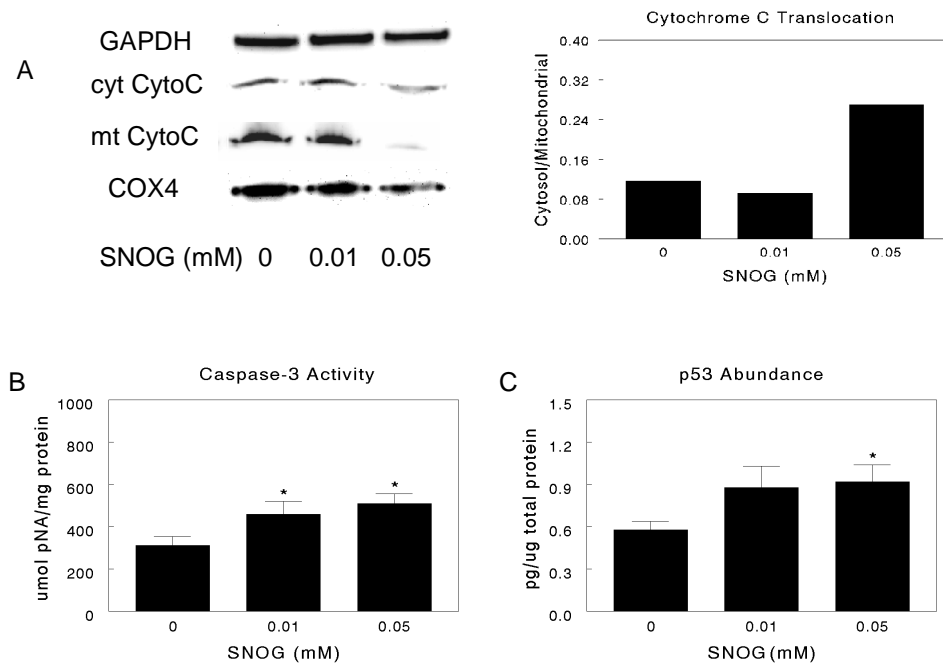
Figure 8 Effect of SNOG on cytochrome C release (A), Caspase-3 activity (B) and p53 expression (C). Cells were treated with 0, 0.01 or 0.05mM SNOG for 24 hours. Data are presented from one representative experiment (A) or as mean ± SEM (n=6) (B,C). *: p<0.05 vs. control.

A   Bax

Bcl-2

GAPDH

SNOG(mM)   0   0.01   0.05   0.5

B
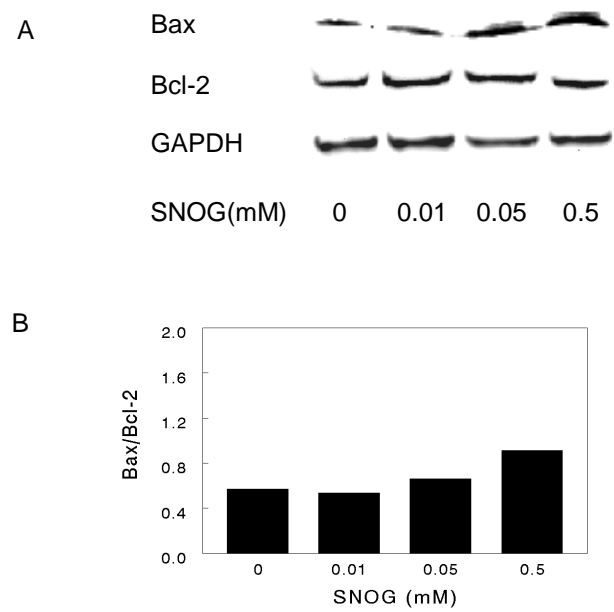
Figure 9 The effect of SNOG on expression of Bax and Bcl-2.   Cells were treated with 0, 0.01, 0.05 or 0.5mM SNOG for 24 hours.   Expression of Bax and Bcl-2 was measured by western blot, with GAPDH as control.   Data presented are from one representative experiment (B).

4.4 NO-induced Chondrocyte Apoptosis was through JNK

The effects of SNOG involved JNK MAP kinase activity. Among the three MAP kinase inhibitors tested, only the inhibitor of JNK was able to block NO-induced apoptosis. Neither PD98059, which inhibits ERK1/2 nor SB203580, which inhibits p38 had an effect (Figure 10). The JNK inhibitor II blocked SNOG-induced apoptosis as shown by MTT assay (Figure 11A), DNA fragmentation (Figure 11B) and TUNEL staining (Figure 11C). Moreover, inhibition of JNK blocked Pi-induced apoptosis in a dose-dependent manner (Figure 11D).
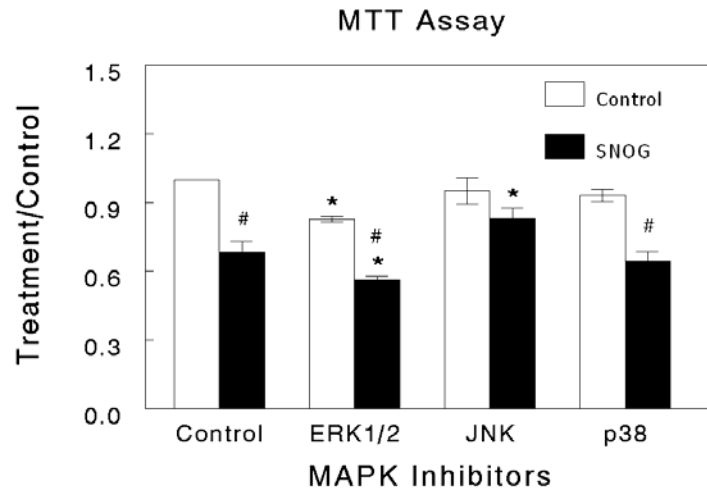
Figure 10 Effect of MAP kinase inhibitors on SNOG-induced apoptosis.   Cells were treated with 10μM PD98059 (ERK1/2), 0.1μM JNK inhibitor II (JNK) or 0.1μM SB203580 for 24 hours.   Half of the cultures also received 0.05mM SNOG as apoptogens.   Cell viability was measured by MTT assay. Data are presented as mean ± SEM (n=6) (B,C).   *: p<0.05 vs. control; #: p<0.05 vs. non-SNOG groups.
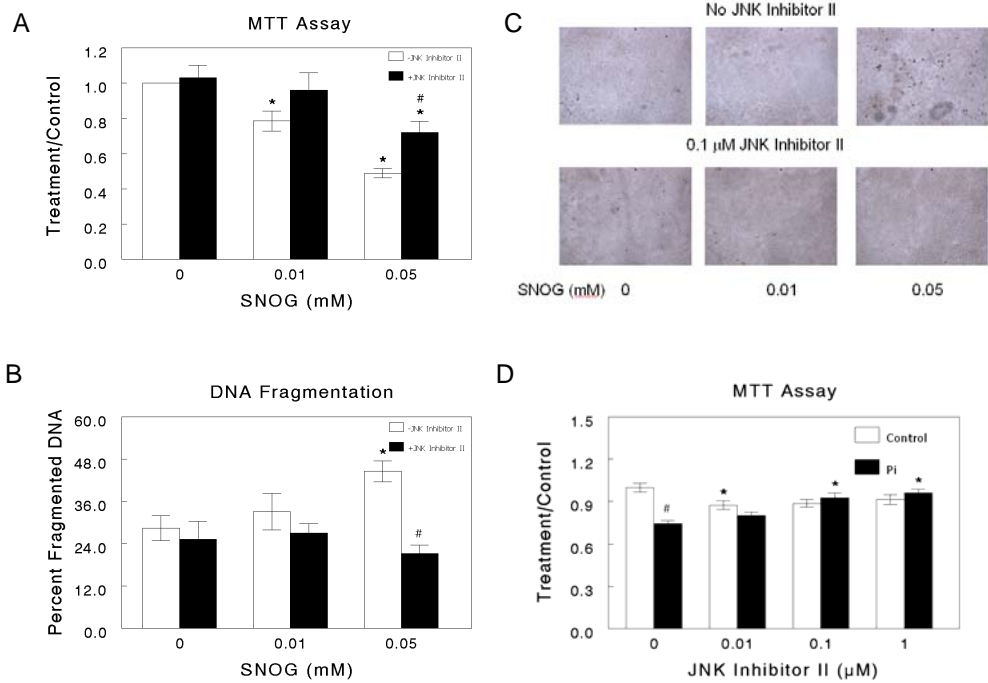
Figure 11 Effect of JNK inhibitor II on chondrocyte apoptosis.   A,B,C: Cells were treated with 0, 0.01 or 0.05mM SNOG for 24 hours.   Half of the cultures also received 0.1μM JNK inhibitor II.   MTT assay (A), DNA fragmentation (B) and TUNEL staining (C) were measured.   Data are presented from one experiment (C) or as mean ± SEM (n=6) (A,B).   *: p<0.05 vs. control; #: p<0.05 vs. non-JNK inhibitor II groups.   D: Cells were treated with 0.01, 0.1 or 1μM JNK inhibitor II for 24 hours.   Half of the cultures also received 7.5mM Pi to induce apoptosis.   Cell viability was measured by MTT assay. Data are presented as mean ± SEM.   *: p<0.05 vs. control; #: p<0.05 vs. non-Pi groups.

4.5 Conclusion

The results above indicate that NO donors dose-dependently increase chondrocyte apoptosis.   This process involves a mitochondria apoptotic pathway that involves elevated p53 and Bax/Bcl-2.   This apoptotic pathway can be blocked by a JNK inhibitor, suggesting that it is JNK-dependent.

Chapter 5

E$_2$-induced Chondrocyte Apoptosis

5.1 Hypothesis and Rationale

The working *hypothesis* of this aim was that estrogen would induce chondrocyte apoptosis in a sex-independent manner, involve nitric oxide and a mitochondrial pathway, similar to Pi-induced apoptosis pathway. The *rationale* was that estrogen induces epiphyseal fusion by exhausting the proliferation potential of growth plate chondrocytes (Weise et al., 2001). The effect of estrogen on growth plate fusion is not sex-dependent (Rodd et al., 2004). High doses of estrogen also induce activation of caspase-3 in the growth plate, indicating involvement of apoptosis (Takano et al., 2008). Estrogen also causes apoptosis in spermatogenic cells through upregulation of NO synthesis (Mishra and Shaha, 2005). Therefore, estrogen may induce chondrocyte apoptosis through NO, similar to Pi.

5.2 E_2 Induced Apoptosis in Growth Plate Chondrocytes

To determine the effect of E_2 on growth plate chondrocyte apoptosis, we employed three different methods: DNA fragmentation, TUNEL staining, and MTT assay. To study the effect of sex and cell type on the action of E_2, we used both male and female resting zone chondrocytes and female growth zone chondrocytes. E_2 caused a dose-dependent increase in apoptosis in both female (Fig. 12A) and male growth plate chondrocytes (Fig. 12B). There was a two-fold increase in DNA fragmentation in cultures treated with 10^{-8}M E_2 in resting zone cells. Growth zone chondrocytes had a similar response to the hormone (Fig. 12C). TUNEL staining showed similar results (Fig. 13A). E_2 caused a dose-dependent decrease in cell viability based on MTT activity (Fig. 13B) and in cell number (Fig. 13C) in both male and female resting zone chondrocytes. The results of these experiments indicate that E_2 dose-dependently increases apoptosis in growth plate chondrocytes. This effect is not sex-dependent. E_2 also increases apoptosis in both resting zone and growth zone chondrocytes.

DNA Fragmentation
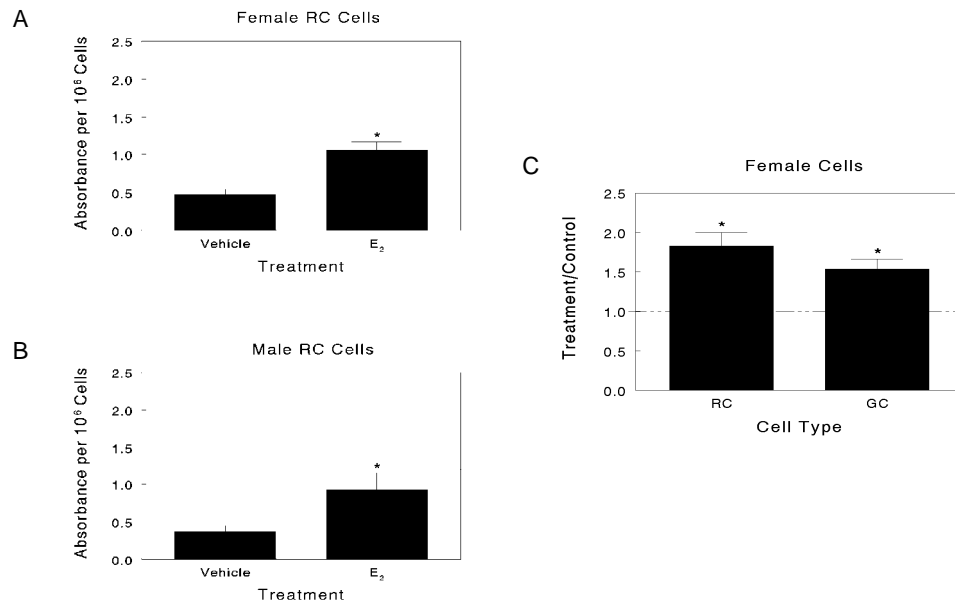
Figure 12 Sex and cell type effect of E_2 on chondrocyte apoptosis.   Male (A) and female (B) RC cells were treated with 10^{-8}M E_2 for 24 hours.   Apoptosis was measured using Cell Death ELISA+.   Data are presented as mean ± SEM (n=6).   *: p<0.05 vs. control; (C) Apoptosis of RC and GC female cells by 10^{-8}M E_2 as measured by Cell Death ELISA+.   Data are presented as mean ± SEM from 6 (RC) or 4 (GC) separate experiments, each of which has n=6.   *: p<0.05 vs. control.
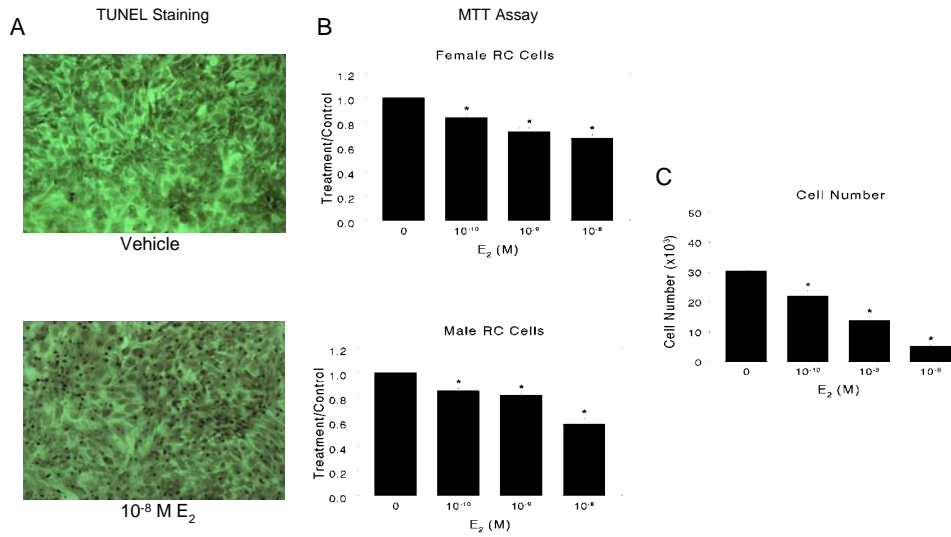
Figure 13 Effect of E_2 on chondrocyte apoptosis (A), viability (B) and cell number (C).   A: female RC cells were treated with 10^{-8}M E_2 for 24 hours.   Apoptosis was measured by TUNEL staining.   B: female and male RC cells were treated with $10^{-10}\sim10^{-8}$M E_2 for 24 hours.   Cell viability was measured by MTT assay.   C: female RC cells treated with $10^{-10}\sim10^{-8}$M E_2 for 24 hour.   Cell number was measured by counting.   Data are presented as mean ± SEM (n=6).   *: p<0.05 vs. control.

5.3 E_2-induced Apoptosis was not NO-dependent

E_2 activates alkaline phosphatase in growth plate chondrocytes (Sylvia et al., 1998).   Since Pi induces resting zone chondrocyte apoptosis (Zhong et al., 2008), E_2 may exert its pro-apoptotic function through increasing local Pi concentration. Pi-induced resting zone chondrocyte apoptosis is NO-dependent.   It can be blocked by either TP508 (Zhong et al., 2008) or PFA (See Chapter 4).   If E_2 does induce apoptosis via elevated Pi production, we should anticipate that E_2-induced apoptosis is also NO-dependent, and can be blocked by TP508 or PFA.   To test this hypothesis, we treated female resting zone chondrocytes with 10^{-10}-10^{-8}M E_2, and measured NO production using the DAN assay.   After 24 hours, E_2 did not increase NO_x content of the conditioned media at any of the concentrations we tested.   At the lowest concentration of E_2, NO_x was reduced compared to control cultures (Fig. 14). Moreover, TP508 had no effect (Table 1). E_2 did not induce apoptosis by a phosphate dependent mechanism.   PFA was able to block E_2's apoptotic effect (Table 1), further indicating that the Pi-induced NO-mediated pathway is not involved.
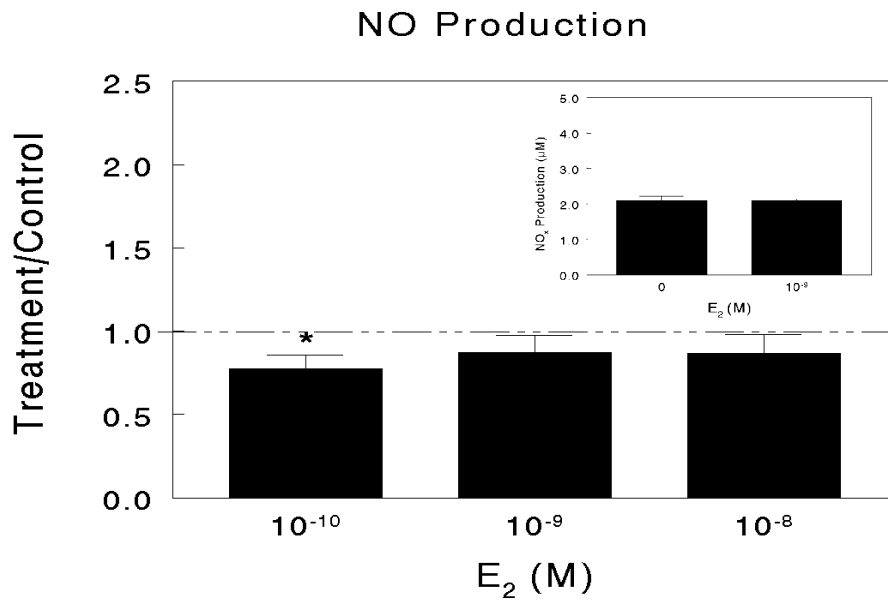
Figure 14 Effect of E_2 on NO production of chondrocytes. Female RC cells were treated with $10^{-10} \sim 10^{-8}$M E_2 for 24 hours. NO production was measured by DAN assay and normalized to protein concentration. Data are presented as mean $\pm$ SEM from 9 separate experiments, each of which has n=6. *: $p<0.05$ vs. control. Inset figure represents a single experiment with control and 10^{-8}M E_2.

Effect of PFA and TP508 on E_2-induced Chondrocyte Apoptosis

	Treatment/Control
Control	1.00
E_2	0.77± 0.03*
PFA	0.96± 0.03#
E_2 + PFA	0.81± 0.04*$

	Treatment/Control
Control	1.00
E_2	0.82± 0.02*
TP508	1.01± 0.01#
E_2 + TP508	0.78± 0.06*$

*: $p < 0.05$ vs. control
#: $p < 0.05$ vs. E_2
$: $p < 0.05$ vs. PFA or TP508

Table 5 Effect of PFA and TP508 on E_2-induced chondrocyte apoptosis.   Cells were treated with 10^{-8}M E_2 for 24 hours.   Half of the cultures also received 1mM PFA or 7μg/ml TP508.   Cell viability was measured by MTT assay.   *: $p < 0.05$ vs. control; #: $p < 0.05$ vs. E_2 group; $: $p < 0.05$ vs. TP508 or PFA group.

5.4 E$_2$-induced Apoptosis was through Caspase Activation and a Mitochondrial

Pathway

In Chapter 4, we have established that Pi-induced apoptotic pathway is through caspase-3 activation and a mitochondrial pathway involving cytochrome C release and up-regulation of Bax/Bcl-2 protein ratio and p53. We tested whether E$_2$-induced chondrocyte apoptosis used the same pathway. E$_2$ caused a modest 1.3-fold increase in caspase-3 activity (Fig. 15A). However, the caspase-3 inhibitor Z-DEVD-FMK almost totally blocked caspase-3 activity in both control and E$_2$ treated groups, and blocked the apoptotic effect of E$_2$ (Fig. 15B). In contrast, blocking caspase-1 activity did not (Fig. 15B). Similarly, the caspase-3 inhibitor Z-DEVD-FMK was able to block the E$_2$-dependent reduction in MTT activity (Fig. 15C).

E$_2$ induced a dose-dependent increase in cytochrome C release from mitochondria, a hallmark of the mitochondrial apoptotic pathway, with 10^{-8}M E$_2$ causing a 5-fold increase in the ratio of cytosol to mitochondrial cytochrome C (Fig. 16A,B). E$_2$ also induced a dose-dependent increase in the expression ratio of Bax/Bcl-2, with 10^{-8}M E$_2$ inducing a 8-fold increase compared with control group (Fig. 16C,D). Expression of p53 increased 2-fold with 10^{-8}M E$_2$ treatment compared with control (Fig. 16E).
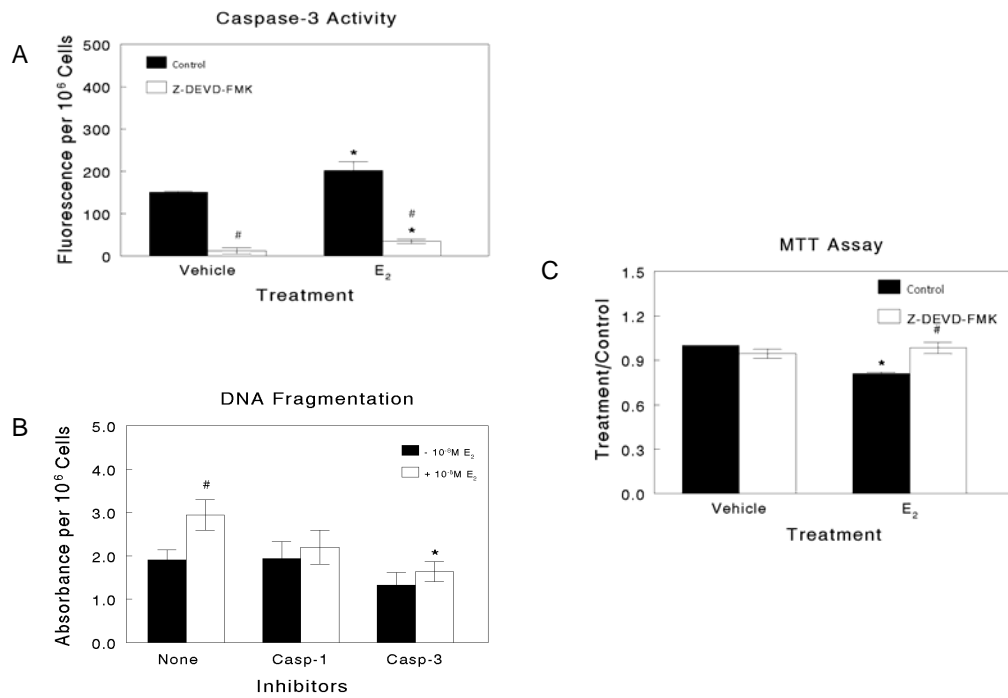
Figure 15 Role of caspase-3 in E_2-induced chondrocyte apoptosis. A: female RC cells were treated with caspase-1 or caspase-3 inhibitors for 24 hours, with or without $10^{-8}M$ E_2 as an apoptogen. Apoptosis was measured by Cell Death ELISA+; B and C: female RC cells were treated with $10^{-8}M$ E_2 to induce apoptosis. Half of the cultures also received a caspase-3 inhibitor. Caspase-3 activity (B) was measured by Caspase-3 fluorescence kit, whereas cell viability (C) was measured by MTT assay. Data are presented as mean ± SEM (n=6). *: p<0.05 vs. control; #: p<0.05 vs. no E_2 groups (B) or no caspase-3 inhibitor groups (A, C).
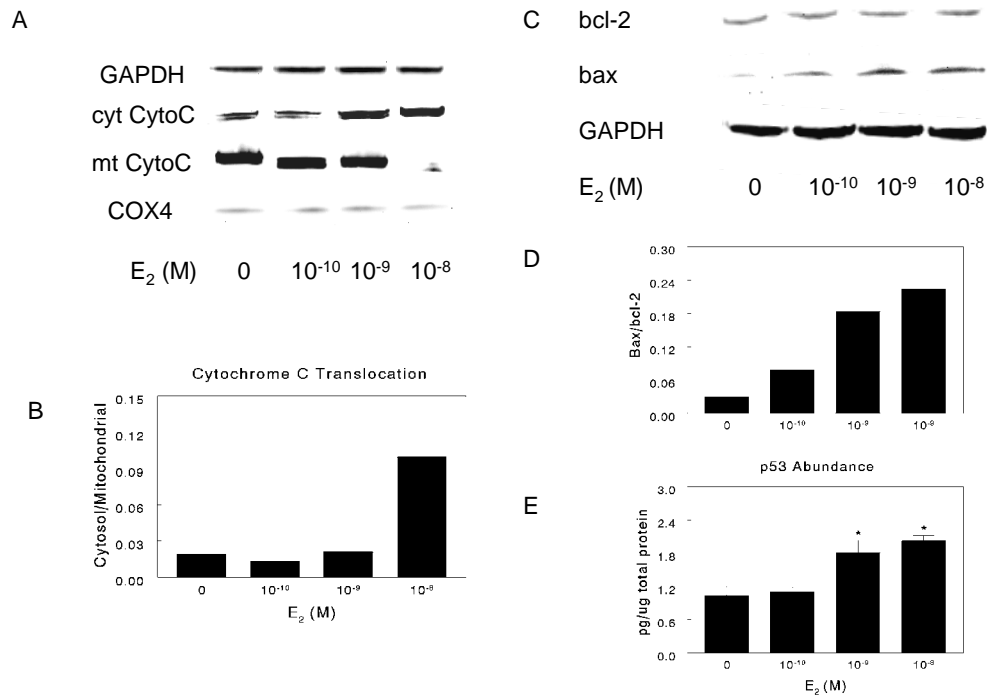
Figure 16 Role of mitochondria in E_2-induced chondrocyte apoptosis. Female RC cells were treated with $10^{-10} \sim 10^{-8}$M E_2 for 24 hours. A and B: cytosolic and mitochondrial fraction were separated by cytochrome c apoptosis kit. Cytochrome C release from mitochondria was measured by Western blot. C and D: expression of Bax and Bcl-2 was measured by western blot, with GAPDH as control. E, expression of p53 was measured using the pan p53 ELISA kit. Data presented are from one representative experiment (B, D) or mean ± SEM (n=6) (E). *: p<0.05 vs. control (E).

5.5 E₂-induced Apoptosis Involved Both Membrane and Nuclear Receptors and not MAP Kinase-dependent

E_2 exerts its effect on chondrocyte differentiation via a rapid, membrane action (Sylvia et al., 1998). To investigate whether the action of E_2 on chondrocyte apoptosis is through membrane or nuclear estrogen receptors, we treated female resting zone chondrocytes with E_2 for 24 hours, with or without co-treatment with ICI182780 (nuclear ER antagonist) or pre-treatment with anti-ERα36 (antibody against membrane ERs). We also used E_2-BSA, an estrogen analog that cannot penetrate the cell membrane, to study the role of membrane receptors. E_2-BSA induced a slight decrease in cell viability of chondrocytes, but the decrease was smaller than that of E_2 alone (Fig. 17A). Both ICI182780 and anti-ERα36 rescued E_2-induced apoptosis. However, ICI182780 was able to exert full recovery (Fig. 17E), whereas anti-ERα36 (Fig. 17B) was only able to partially rescue the cells. Caspase-3 activity was regulated in a similar manner (Fig. 18A,B,E), although anti-ERα36 blocked all of the effect of E_2 on this enzyme.

The action of E_2 on chondrocyte proliferation and differentiation both involve activation of ERK1/2 and p38 in a PLC-dependent pathway (McMillan et al., 2006). Moreover, from the previous chapter, Pi-induced chondrocyte apoptosis involves JNK. To test the effect of MAP kinases on E2-induced apoptosis, we tested the ability of ERK1/2 inhibitor PD98059, p38 inhibitor SB203580 and JNK inhibitor II SP600125 to block E2-induced apoptosis. We also tested the effect of a PLC inhibitor, U73122,

and the PLA2 inhibitor quinacrine. The ERK1/2 inhibitor PD98059, the p38 inhibitor SB203580 and JNK inhibitor II did not rescue E_2-induced apoptosis (Fig. 17F). PD98059 and JNK inhibitor II themselves caused apoptosis indicating that both MAPKs modulate cell survival (Fig. 17F). Neither the PLC inhibitor U73122 nor the PLA$_2$ inhibitor quinacrine rescued E_2-induced apoptosis, as judged both by MTT assay (Fig. 17C,D) and caspase-3 activity (Fig. 18C,D). However, both compounds caused a dose-dependent increase in apoptosis, in a level comparable to E_2-induced apoptosis (Fig. 17C,D,18C,D).

In summary, these results show that E_2 use both membrane and nuclear receptor to cause chondrocyte apoptosis. This pathway is not MAP kinase-dependent.

MTT Assay
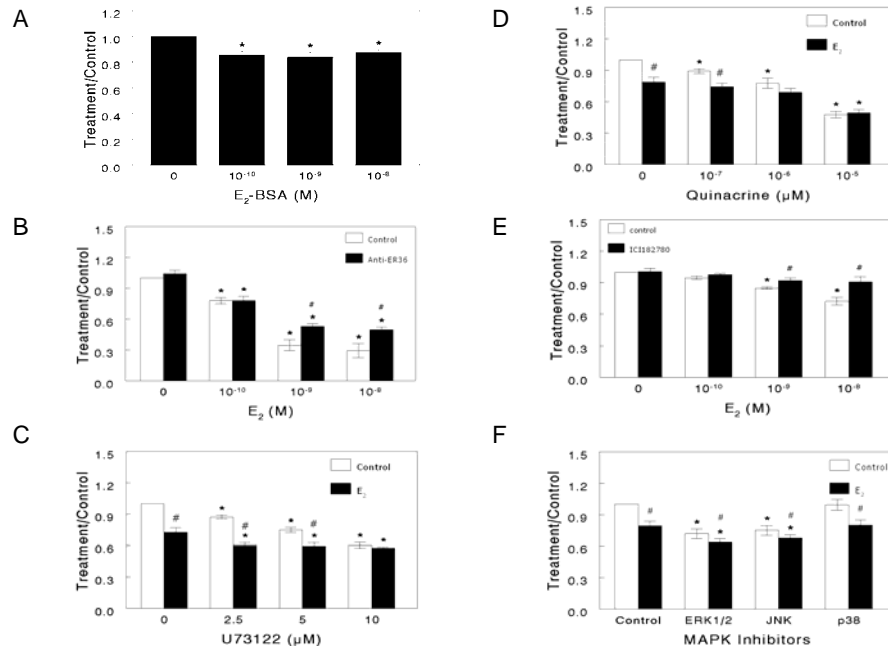
Figure 17 Role of estrogen receptors PLA$_2$, PLC and MAP kinases in E$_2$-induced chondrocyte apoptosis. A, cells were treated with 10^{-10}~10^{-8}M E$_2$-BSA for 24 hours.   B, cells were treated with 10^{-10}~10^{-8}M E$_2$ for 24 hours with or without pre-treatment with antibody vs. ERα36;   C, cells were treated with 2.5 to 10μM PLC inhibitor U73122, half of the cultures also received 10^{-8}M E$_2$;   D, cells were treated with 10^{-7} to 10^{-5}M PLA$_2$ inhibitor quinacrine, half of the cultures also received 10^{-8}M E$_2$;   E, cells were treated with 10^{-10}~10^{-8}M E$_2$ for 24 hours, half of the cultures also received estrogen antagonist ICI 182780;   F, cells were treated with inhibitors vs. ERK1/2 (PD98059), JNK (JNK inhibitor II) and p38 (SB203580), together with 10^{-8}M E$_2$ for 24 hours.   Cell viability was measured by MTT assay;   *: p<0.05 vs. control group; #: p<0.05 vs. no no anti-ERα36 groups (B), ICI182780 groups (E), or no E$_2$ groups (C, D, F).

Caspase-3 Assay
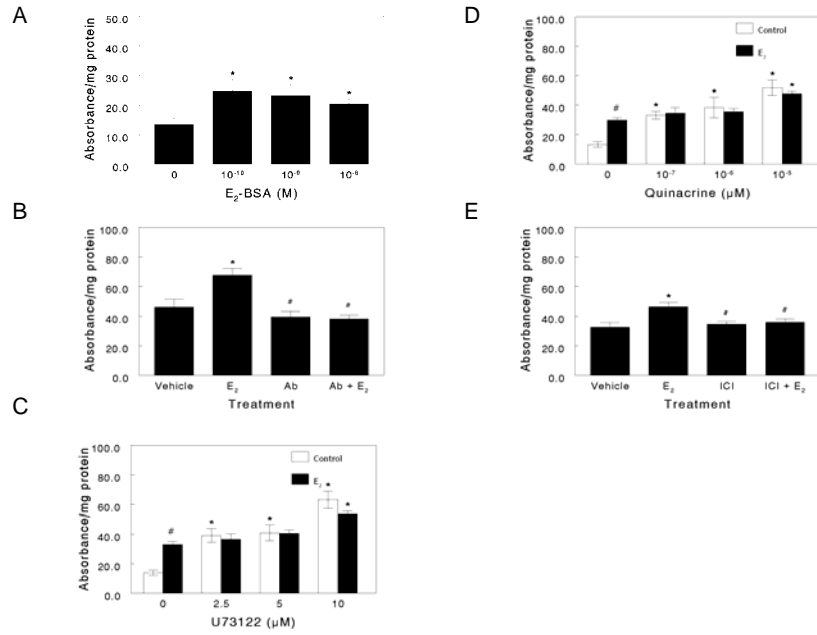
Figure 18 Role of estrogen receptors, PLA_2, and PLC on E_2-induced chondrocyte apoptosis. A, cells were treated with $10^{-10} \sim 10^{-8}$M E_2-BSA for 24 hours. B, cells were treated with 10^{-8}M E_2 for 24 hours, half of the cultures were also pre-treated with antibody vs. ERα36 (Ab); C, cells were treated with 2.5 to 10μM PLC inhibitor U73122, half of the cultures also received 10^{-8}M E_2; D, cells were treated with 10^{-7} to 10^{-5}M PLA_2 inhibitor quinacrine, half of the cultures also received 10^{-8}M E_2; E, cells were treated with 10^{-8}M E_2 for 24 hours, half of the cultures also received estrogen antagonist ICI 182780 (ICI); apoptosis was measured as caspase-3 activity; *: $p < 0.05$ vs. control group; #: $p < 0.05$ vs. E_2 groups (B, E), or no E_2 groups (C, D).

5.6 Conclusion

The results above indicate that although E_2 also dose-dependently induces chondrocyte apoptosis in a mitochondrial pathway, it differs from Pi in that E_2-induced apoptosis pathway does not involve NO or JNK. This pathway also requires both membrane and nuclear ER.

Chapter 6

Discussion and Conclusion

6.1 Pi-induced Apoptotic Pathway

Apoptosis in the mammalian growth plate resting zone occurs relatively infrequently (Roach, 1997), whereas hypertrophic chondrocytes in the growth zone routinely undergo physiological apoptosis during terminal differentiation. In avians, apoptosis in the growth zone is via a mitochondrial pathway (Teixeira et al., 2007), involving elevated extracellular inorganic phosphate and NO production (Mansfield et al., 1999; Teixeira et al., 2001). Pi also induces apoptosis in rat growth plate chondrocytes (Zhong et al., 2008; Zhong et al., 2009). In vitro, both growth zone and resting zone chondrocytes are affected (Zhong et al., 2008; Zhong et al., 2009). The concentrations of Pi that caused significant apoptosis in the present study were similar to levels found near the calcified portion of growth plate (Shapiro and Boyde, 1984), whereas extracellular Pi content of the resting zone cartilage is comparatively low. This suggests that maintaining low extracellular Pi is a survival mechanism for cells in the reserve zone.

Pi induced apoptosis in rat costochondral resting zone cells by a mechanism comparable to that reported for the avian growth zone chondrocytes. Pi caused an

increase in NO production and NO acted on the cells via a pathway involving JNK

MAPK.   Caspase-3 activity was increased as was abundance of p53, the Bax/Bcl-2

ratio, and release of cytochrome C from the mitochondria.   Blocking the Pi

transporter with PFA prevented NO production as well as downstream NO-dependent

actions resulting in apoptosis, confirming that Pi uptake was responsible for inducing

apoptosis and the mechanism involved required NO.   Morever, we used NO synthase

inhibitors and an NO synthase knockout animal model to demonstrate that NO

production is necessary in Pi-induced chondrocyte apoptosis.   We also employed NO

donors NOC-18 and SNOG to demonstrate that this NO-dependent Pi-induced

chondrocyte apoptotic pathway signals through JNK, p53, Bax/Bcl-2, cytochrome C

release and caspase-3 activation.

Pi-induced NO production occurs via an eNOS-dependant mechanism.

Previously we reported that the PKC inhibitor chelerythrine induced apoptosis in

resting zone chondrocytes by an NO-dependent pathway role and that iNOS was

responsible for NO production we observed (Zhong et al., 2008). The results also

implicated eNOS but to a lesser extent.   In the present study, we used L-NAME,

which inhibits both forms of the enzyme.   Most of the apoptotic effect of Pi was

blocked, confirming the critical role of NO, but still not resolving the synthase

responsible. Cells from mice lacking eNOS failed to exhibit Pi-induced apoptosis,

indicating that eNOS was involved.   In addition, we found that Pi increased eNOS

protein, suggesting that Pi might increase NO production by increasing the amount of

NO synthases.   We did not find either iNOS or nNOS in control or Pi-treated cells,

suggesting that these two NO synthases might not play any role in Pi-induced apoptosis. However, elevated iNOS expression may have happened much earlier than the time point (24 hours) we checked. In addition, eNOS-/- cells exhibited basal production of NO, indicating other NO synthases might exist in chondrocytes.

The participation of eNOS in the apoptotic response to Pi is not consistent with the report that eNOS blocks apoptosis in mouse endothelial cells (Hoffmann et al., 2001). This suggests that the same stimulus may have different effects on the cell fate in different cell types. It should be noted that in both endothelial cells and chondrocytes, the effect of eNOS on apoptosis is physiological and beneficial to the organism.

Elevated p53 levels correlates with diminished cell survival (Bartek et al., 1990). p53 is able to induce apoptosis in cancer cells via a mitochondrial apoptotic pathway involving Bax expression and cytochrome C release from mitochondria (Chipuk et al., 2003). In this study, we have also found that NO induced an increase in p53, Bax and cytochrome C release, indicating NO-induced apoptosis involves increasing p53 expression, which induces a mitochondrial apoptotic pathway. We have previously reported that lysophosphatidic acid (LPA) protects resting zone chondrocytes from apoptosis by decreasing p53 and Bax expression, suggesting this pathway is pivotal in regulating resting zone chondrocyte apoptosis (Hurst-Kennedy et al., 2009).

Our finding that NO induces chondrocyte apoptosis via a mitochondrial

apoptotic pathway is in accord with previous reports in fetal chicken growth plate (Teixeira et al., 2007), human articular chondrocytes (Cherng et al., 2008) and rabbit articular chondrocytes (Wang et al., 2007), indicating that the mitochondrial apoptosis pathway induced by NO may be evolutionarily conserved across vertebrates (aves vs. mammals) and cartilage tissues (growth plate vs. articular cartilage). The concentration of SNOG (0.05mM) that we used results in a level of NO_x in the media similar to the level we have previously found to be induced by 7.5mM Pi (Zhong et al., 2008; Zhong et al., 2009), indicating that it is physiologically relevant.

MAP kinases JNK and p38 have been associated with apoptosis induced by multiple stress factors in a wide range of cells (Strniskova et al., 2002). In our rat growth plate model, we have found that p38 mediates the rapid membrane response to estrogen (McMillan et al., 2006). In our current study, we have found that inhibiting JNK, but not p38 blocks NO-induced chondrocyte apoptosis. This result is in accord with a previous report in human articular chondrocytes that sodium nitroprusside (SNP), another NO donor, induces JNK phosphorylation (Cherng et al., 2008). However, in rabbit articular cartilage, p38 seems to be the key regulator of NO-induced apoptosis (Wang et al., 2007). These results show that in different species, different signaling pathways may be employed to conduct NO-induced apoptosis.

The results from this study delineate the signaling pathway that leads to Pi-induced apoptosis in resting zone chondrocytes. This study allows us to further

understand the mechanisms that regulate the development of growth plate. It also suggests therapeutic options concerning growth plate defects resulting from dysfunctions in this pathway. Moreover, knowledge obtained from this study can also be applied to the treatment of bone fractures and osteoarthritis, given the importance of endochondral bone formation in these processes. For example, blocking Pi-induced apoptosis by inhibiting components in this pathway may help increase the population of resting zone chondrocytes, resulting in more cartilage formation, as previously shown in the case of bone fracture healing ability of TP508 (Zhong et al., 2008).

6.2 E_2-induced Apoptotic Pathway

E_2 induces a rapid increase in PKC activity via a G protein-coupled PLC pathway that is functional only in cells from female rats (Sylvia et al., 2000). This process is membrane-associated (Sylvia et al., 2001b) and mediates the effects of E_2 on chondrocyte differentiation via signaling pathway that involves ERK1/2 and p38 MAP kinase (McMillan et al., 2006). The failure of inhibitors of this pathway to block E_2-dependent apoptosis suggests that E_2 regulates chondrocyte apoptosis by different mechanisms than are used to regulate chondrocyte differentiation. The results also strongly support the hypothesis that the E2-dependent PKC pathway is involved in cell survival, based on the observation that inhibition of components of the pathway led to increased apoptosis. Interestingly, the E2-dependent apoptotic pathway is functional in male cells further indicating that the mechanism involved is distinct from those that mediate chondrocyte differentiation.

This study shows that E_2 regulates apoptosis in rat costochondral growth plate chondrocytes, affecting cells from the resting zone as well as cells in the growth zone. E_2 caused dose-dependent increases in DNA fragmentation, confirmed by TUNEL staining, and decrease in cell viability and, ultimately in cell number. Although hypertrophic chondrocytes in the growth zone routinely undergo physiological apoptosis, apoptosis in resting zone chondrocytes occurs less frequently (Roach, 1997). Others have reported that a high dose of E_2 induced elevated caspase-3 activity in the resting zone as well as growth zone of rabbit growth plates (Takano et al., 2008), suggesting our observations that apoptosis may be initiated in less terminally differentiated cells. The resting zone is the reservoir of chondrocytes that form growth plate cartilage, and regulation of cell kinetics in this zone has important implications in physiological functions (Zhong et al., 2008).

Estrogen causes growth plate senescence in rabbits (Schrier et al., 2006), and is responsible for the diminishment and eventual disappearance of mammalian growth plate in both sexes (Rodd et al., 2004). Although rodents do not normally undergo epiphyseal fusion at adulthood, supraphysiological levels of estrogen induce epiphyseal fusion in mice (Chagin et al., 2004), suggesting that rodents' failure to fuse their growth plates may be due to an evolutionary change in either estrogen level or sensitivity of growth plate chondrocytes to estrogen. In either case our results suggest that mammal epiphyseal fusion may be caused by direct regulation of resting zone chondrocytes by E_2-induced apoptosis.

In our current study, we also explored whether E_2's apoptotic effect is through

nuclear or membrane receptors. A previous study in mice shows that the growth plates of ERα/β knockout mice do not fuse even with supraphysiological estrogen exposure (Chagin et al., 2004), suggesting nuclear receptors are involved in estrogen-induced apoptosis. However, the study did not examine whether membrane receptors were also knocked out, so a membrane receptor pathway cannot be ruled out. Although estrogen's effects on proliferation and differentiation of resting zone chondrocytes are membrane-mediated and not inhibited by the estrogen receptor antagonist ICI 182780 (Sylvia et al., 2001b), the effect of E_2 on apoptosis is fully blocked by ICI 182780, indicating that membrane receptors are necessary in mediating E_2-induced apoptosis. However, E_2-BSA, which cannot penetrate cell membrane, also causes apoptosis, though in a manner less significant than E_2 of comparable concentration. Moreover, pretreatment of cells with anti-ERα36, a specific antibody that blocks the estrogen membrane receptor ERα36, was also able to rescue E_2-induced apoptosis. Taken together, E_2-induced apoptosis requires both membrane and nuclear receptor pathways. PKC inhibitors such as chelerythrine and tamoxifen also induce apoptosis in resting zone chondrocytes (Zhong et al., 2008), therefore it is difficult to assess PKC's role in E_2-induced apoptosis. However, since E_2-induced PKC activation is female-only (Sylvia et al., 1998) and, whereas E_2-induced apoptosis is not sex-dependent, E_2 is likely to induce apoptosis in a PKC-independent pathway.

Having established the ability of E_2 to induce chondrocyte apoptosis, we wanted to explore its pathway. E_2 is able to induce alkaline phosphatase activation (Sylvia et al., 1998). Increased alkaline phosphatase is associated with chondrocyte

hypertrophy and apoptosis (Roach et al., 1995b; Roach and Erenpreisa, 1996). Moreover, we have shown that elevated phosphate can directly induce apoptosis in resting zone chondrocytes by up-regulating NO production (Zhong et al., 2009). These results are in accordance with previous studies showing that increased NO production and subsequent changes of mitochondrial activity are normally associated with both pathological and physiological chondrocyte apoptosis (Teixeira et al., 2001; van den Berg, 2001). Moreover, estrogen also causes apoptosis in spermatogenic cells through upregulation of nitric oxide synthesis (Mishra and Shaha, 2005). In this study we examined whether estrogen-induced apoptosis in resting chondrocytes also follows this pathway. However, estrogen decreases NO production, indicating that this apoptotic pathway is independent on NO production. Estrogen-induced apoptosis is also not blocked by TP508 or PFA, which further indicates that estrogen does not induce apoptosis via a phosphate-related mechanism. In NO-dependent chondrocyte apoptotic pathways, inhibition of JNK activity blocks apoptosis (manuscripts preparation). In our study, all three MAP kinase inhibitors, including a JNK inhibitor, were not able to block estrogen's effect, further indicating that estrogen acted in an NO-independent manner. Our results also show that estrogen-induced apoptosis in chondrocytes is via a mitochondrial mechanism as judged by cytochrome C release from mitochondria and caspase-3 activation, indicating that E_2 and Pi-induced apoptosis pathways converge at this point. Moreover, we have also examined the effect of E_2 on p53 expression and Bax/Bcl-2 expression ratio. E_2 induces apoptosis in breast cancer cells in p53-mediated pathways (Dunphy et al., 2008; Shcherbakov et al.,

2006). E_2 also induces p53 activation in osteoblasts (Chandar et al., 2004). p53 is able to induce apoptosis in cancer cells via the mitochondrial apoptotic pathway involving Bax expression and cytochrome C release from mitochondria (Chipuk et al., 2003). We found that E_2 elevated both Bax/Bcl-2 protein ratio and p53 protein expression in resting zone chondrocytes in 24 hours, implying E_2 induces mitochondrial apoptotic pathway via up-regulating p53 which in turn induces Bax activation and that leads to cytochrome C release and apoptosis.

6.3 Conclusion and Future Study

The results from this study delineate the signaling pathways that lead to both Pi-induced and E_2-induced apoptosis in resting zone chondrocytes (Figure 19). Both pathways signal through p53, elevation of which leads to upregulation of Bax. Bax causes cytochrome C release from mitochondria, which subsequently induces caspase-3 activation, the executioner caspase which carries out the downstream apoptosis events. However, these two pathways differ upstream of p53. Pi-induced apoptosis involves JNK activation and NO production, whereas E_2-induced apoptosis does not involve these two. This study has demonstrated both the versatility of cell signaling pathways that induce apoptosis and the conservation of the execution phase of apoptosis in our model.

This study has laid the foundation for potential more valuable future research. Although we have shown that both E_2 induces chondrocyte apoptosis by increasing p53 expression, the signaling pathway leading to p53 is still unclear, and the

exploration of this pathway may give us more insights into the crucial role of p53 in physiology and pathology of cartilage. Moreover, do the membrane and nuclear receptor of E_2 work independently or interacting with each other to carry out the apoptotic effect of E_2? As for Pi-induced apoptotis, the question how Pi induces NO production by eNOS also begs our answer. Last but not least, we should also ponder how to apply the discoveries of this study to cartilage tissue engineering. Preferably, blocking some components of the described apoptotic pathway has the potential to improve the chondrocyte cellularity in tissue engineering constructs.

This study allows us to further understand the mechanisms that regulate the development of growth plate, such as the E_2-induced epiphyseal fusion. It also suggests therapeutic options concerning growth plate defects resulting from dysfunctions in these pathways. Moreover, knowledge obtained from this study can also be applied to the treatment of bone fractures and osteoarthritis, given the importance of endochondral bone formation in these processes. For example, blocking Pi-induced apoptosis by inhibiting components in this pathway may help the build-up of the resting zone chondrocyte population, resulting in more cartilage formation, as previously shown in the case of bone fracture healing ability of TP508 (Zhong et al., 2008).
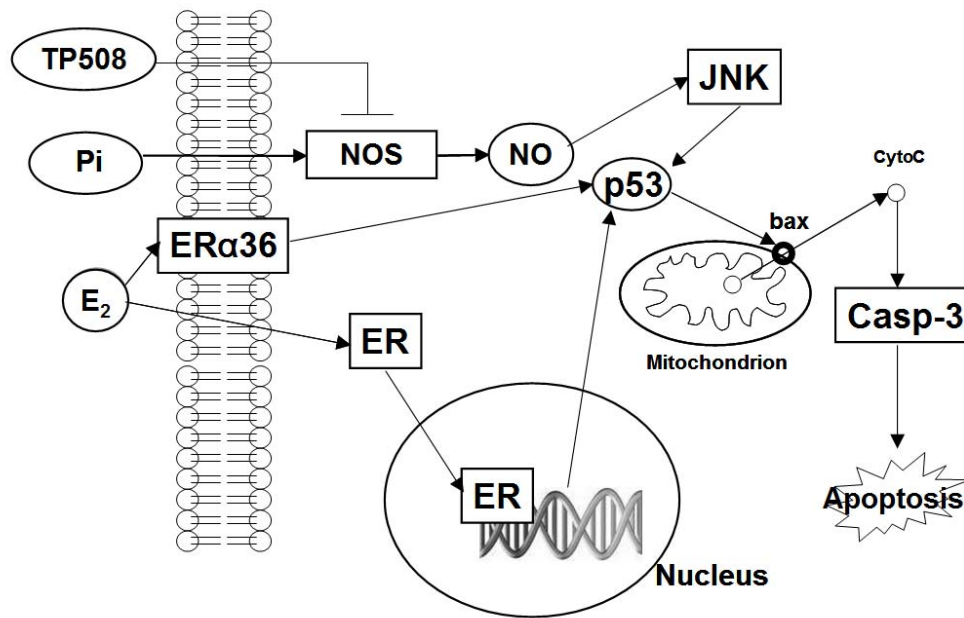
Figure 19 Overview of Pi-induced and E_2-induced apoptotic pathway in mammalian growth plate resting zone chondrocytes. Both pathways involve a mitochondrial pathway that works through upregulation of p53 and Bax expression, cytochrome C release and caspase-3 activation. However, Pi-induced apoptosis goes through both NO and JNK, while E_2-induced apoptosis does not involve either.

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