이학박사학위논문

Dehydrodiconiferyl Alcohol의 항염증 및 항골다공증 활성에 대한 분자 기전 연구

Molecular Characterization of Anti-Inflammatory and Anti-Osteoporotic Activities of Dehydrodiconiferyl Alcohol

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이 논문을 이학박사 학위논문으로 제출함

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이원우의 이학박사 학위논문을 인준함

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Abstract

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from *Cucurbita moschata*. Synthetic DHCA has previously been shown to contain anti-adipogenic, anti-oxidative stress and anti-inflammatory activities. In an effort to understand the underlying mechanisms of such multiple bioactivities of this lignan molecule, my thesis research was focused on the effects of DHCA on IL-17-mediated inflammation and also on its potential estrogenic activities, both *in vitro* and *in vivo*.

The effects of DHCA on IL-17-mediated inflammation were investigated using HaCaT keratinocyte cell line. DHCA significantly inhibited the IL-17-mediated cell proliferation and suppressed the expression of various inflammatory mediators, such as TNF-α, IL-6, IL-1β and various chemokines by inhibiting p38 MAPK signaling pathway. Consistent with these in vitro data, in the imiquimod-induced psoriasis-like skin inflammation mouse model, DHCA ameliorated psoriatic symptoms, histological phenotypes and expression of various inflammatory mediators. Data from immunohistochemical analysis and ex vivo culture experiments suggested that DHCA reduced the infiltration of IL-17 producing inflammatory cells by suppressing various chemokines such as CXCL1, CXCL8 and CCL20.

Being a lignan molecule, DHCA is a member of the phytoestrogen family. Therefore, it was investigated whether DHCA contains anti-osteoporotic activities similar to estrogen. The effects of DHCA were studied on RANKL-induced osteoclastogenesis using RAW264.7 pre-osteoclast cell line. DHCA effectively inhibited the RANKL-induced differentiation and function of osteoclast in a dose-dependent manner. DHCA also suppressed the expression of various osteoclastogenic genes, including NFATc1, TRAP, c-Fos, DC-STAMP, MMP-9, and Cathepsin K, through the inhibition of NF-κB and p38 MAPK signaling pathways. These anti-osteoclastogenic effects of DHCA were suppressed when cells were transfected with siRNAs for AMPKα1 or ERα, whereas ERβ siRNA did not have any effect.

The effects of DHCA on BMP-2-induced osteoblastogenesis were also studied using MC3T3-E1 pre-osteoblast cell line. DHCA promoted BMP-2-induced differentiation of

osteoblast in a dose-dependent manner. This lignan molecule further up-regulated the BMP-2 mediated activation of Smad1/5/9 and AMPK signaling pathways, the expression of RUNX2 and subsequently that of ALP, osteocalcin and OPG. Gene knockdown analysis, involving specific siRNAs for ERα or ERβ, indicated that DHCA might interact with either ERα or ERβ to promote the BMP-2-induced osteoblast differentiation.

Above data indicated that DHCA might produce anti-osteoporotic activities through its agonistic effect on estrogen receptor. When an ovariectomized mouse model was used, DHCA indeed improved a variety of bone morphometric parameters as determined by 3D-structure analysis. DHCA also reduced the blood level of NTx and CTx, biochemical markers for bone degradation which also regulate the expression of osteoclastogenic and osteoblastogenic genes in the bone marrow.

Together with our previous findings, data from my thesis work demonstrated that DHCA had a wide range of bioactivities including anti-adipogenic, anti-oxidative stress, anti-inflammatory, anti-osteoclastogenic and osteoblastogenic activities. Such multiple bioactivities of DHCA could be best explained if this molecule acts like estrogen. Data from molecular docking simulation suggested that DHCA could bind to both ERα and ERβ with a similar binding pose to estrogen. Furthermore, the 2D ligand-receptor interaction diagram showed that intermolecular forces and MM-GBSA binding energy were analogous to the case of estradiol. Indeed, results from estrogen receptor competition assay indicated that DHCA could efficiently bind to ERα and ERβ.

In conclusion, high therapeutic effects of DHCA observed in psoriasis and osteoporosis mouse models could be explained with DHCA acting as an estrogen receptor agonist, and thus producing anti-inflammatory, anti-osteoclastogenic and osteoblastogenic activities. Taken together, with previous findings, DHCA may be developed as a safe and effective therapeutic agent for the treatment of various diseases where inflammation and/or estrogen play prominent role(s).

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ABBREVIATION

Chapter I

Introduction

1. Background information

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from water-soluble extracts of *Cucurbita moschata* in our laboratory(Lee et al. 2012). Our group previously reported that DHCA exerts a wide range of biological activities, including anti-adipogenic(Lee et al. 2012), anti-inflammatory(Lee, Choi, and Kim 2015) and anti-oxidative stress(Lee and Kim 2014) effects by regulating C/EBPβ (CCAAT/enhancer-binding protein beta), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AMPK (AMP-activated protein kinase) signaling pathways, respectively. However, at the time when I initiated my thesis research, it was not clear whether DHCA could be used for any particular pathologic conditions.

Based on those biological activities associated with DHCA, my thesis research initially focused on testing whether DHCA could target 3 different, but sharing some common pathologic mechanisms, and attempted to unravel a detailed molecular mechanism that may underlie the “therapeutic” effects of DHCA. Because DHCA was classified as a member of phytoestrogens based on its structure, I also investigated the effects of DHCA on the regulation of bone remodeling process including osteoblastogenesis and osteoclastogenesis. In an effort to understand the mechanisms of actions of DHCA, molecular docking simulation and estrogen receptor competition assay were performed. Based on the data obtained from these experiments, the therapeutic potential of DHCA was tested in mouse models of psoriasis and osteoporosis, respectively.

2. Phytoestrogens

Phytoestrogens are plant-derived xenoestrogens which are not made by human endocrine system, but consumed by eating phytoestrogenic plants (Table 1). It has been suggested that phytoestrogens may have various protective activities against diverse health disorders, such as prostate, breast, bowel, and other cancers, cardiovascular disease, brain

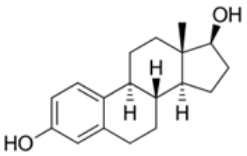
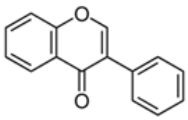
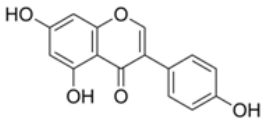
	Estrogen	Phytoestrogen
Structure		 Isoflavone  Genistein
Efficacy	Female sex hormone Induces the development of sex-organ Anti-aging, Anti-oxidative stress and Anti-inflammatory activities	Female sex hormone-like Induces the development of sex-organ Anti-aging, Anti-oxidative stress and Anti-inflammatory activities Preventive effect on tumor
Side Effect	Increasing to be in danger of breast cancer, the endometrium when it used for a long time	No side effects
Source	Synthesis	Natural (from plants)

Table 1. Differences and similarities between estrogen and phytoestrogen

function disorders and osteoporosis(Ibarreta, Daxenberger, and Meyer 2001). Phytoestrogens are not considered as nutrients, given that their lack in the diet does not produce any characteristic deficiency syndrome and they do not participate in any essential biological function(Bacciottini et al. 2007).

Phytoestrogens mainly belong to a large group of substituted natural phenolic compounds: coumestans, prenylflavonoids and isoflavones are the three phytoestrogens in this class containing the highest level of estrogenic activities(Pan et al. 2015). The best-researched are isoflavones, which are commonly found in soy and red clover(Xu et al. 1998). Lignans have also been identified as phytoestrogens, although they are not flavonoids(Lampe 2003).

Because of their structural similarity with estrogen (17β-estradiol), phytoestrogens have the ability to cause estrogenic and/or anti-estrogenic effects, by sitting in and blocking receptor sites against estrogen(Rosenblum et al. 1993). Phytoestrogens exert their effects primarily through binding to ERs (estrogen receptors). There are two variants of estrogen receptors, alpha (ERα) and beta (ERβ), and many phytoestrogens display somewhat higher affinity for ERβ compared to ERα(Dechering, Boersma, and Mosselman 2000). In addition to their interaction with ERs, phytoestrogens may also modulate the concentration of endogenous estrogens by binding to or inactivating certain enzymes, and may affect the bioavailability of sex hormones by depressing or stimulating the synthesis of SHBG (sex hormone-binding globulin)(Pino et al. 2000).

Emerging evidence show that some phytoestrogens bind to transactivate PPARs (peroxisome proliferator-activated receptors)(Jungbauer and Medjakovic 2014). In vitro studies show an activation of PPARs at concentrations above 1μM, which is higher than the activation level of ERs. Studies have shown that both ERs and PPARs influence each other and therefore induce differential effects in a dose-dependent manner(Bonofiglio et al. 2005). For example, the final biological effects of genistein are determined by the balance among these pleiotrophic actions(Nagaraju, Zafar, and El-Rayes 2013).

3. Psoriasis

Psoriasis is an incurable, chronic inflammatory skin disease that produces plaques of thickened, scaling skin on elbows, knees and scalp(Naldi and Rzany 2009). The spectrum of psoriasis ranges from mild with limited involvement of small areas; to large, thick plaques; to red inflamed skin affecting the entire body surface(Raut, Prabhu, and Patravale 2013). Although any area of the human body may be affected, psoriasis tends to be more common at sites of friction, scratching or abrasion(Carrascosa et al. 2014). The dry flakes of skin scales may result from the excessively rapid proliferation of keratinocytes, which is triggered by inflammatory mediators produced by specialized white blood cells such as neutrophils and lymphocytes(Valdimarsson et al. 1986).

Psoriasis affects all races and both sexes(Parisi et al. 2013). Although psoriasis can be seen in people of any age, most commonly patients are first diagnosed in their early adult years. Currently, more than 2-3% of the world's population are estimated to be suffering from psoriasis(Parisi et al. 2013). The quality of life in patients with psoriasis is often diminished because of the appearance of their skin(Augustin and Radtke 2014). Recently, it has been reported that people with psoriasis are more likely to suffer diabetes, cardiovascular disease and a variety of other inflammatory diseases(Armstrong, Harskamp, and Armstrong 2013). This may reflect their inability to control inflammation.

Pathophysiology : While the exact cause of psoriasis is yet to be identified, it has been reported that a combination of various elements, including genetic predisposition and environmental factors, contribute to the pathogenesis of psoriasis(Dika et al. 2007). Among many different factors, defects in immune regulation (for example, T lymphocytes target normal cells instead of attacking foreign substances) and the regulation of inflammatory responses are

thought to play a important role(s) in the progression of psoriasis (Figure 1)(Hueber and McInnes 2007).

Psoriasis is characterized by an abnormally excessive and rapid growth of the epidermal layer of the skin(Valdimarsson et al. 1986). Abnormal proliferation of keratinocytes results from the sequence of pathological events in psoriasis. Keratinocytes are replaced every 3–5 days in psoriasis rather than the usual 28-30 days(Weinstein and Frost 1968). These changes are considered to cause the pre-maturation of keratinocytes, induced by the immune cells such as dendritic cells, macrophages, neutrophils and T cells. Activated immune cells migrate from the dermis to the epidermis, and secrete pro-inflammatory cytokines such as TNF-α (tumor necrosis factor alpha), IL-1β (Interleukin 1 beta), IL-6 (Interleukin 6), IL-17 (Interleukin 17) and IL-22 (Interleukin 22)(Lowes, Suarez-Farinas, and Krueger 2014). In response to these cytokines from immune cells, keratinocytes secrete various inflammatory mediators which stimulate other inflammatory cells to infiltrate to the problematic area(s), and activate additional inflammatory responses involved in the development of psoriasis(Lowes, Suarez-Farinas, and Krueger 2014).

Recently, it has been reported that the IL-17 signaling pathway plays a major role(s) in the progression of psoriasis(Malakouti et al. 2015). Analysis of biopsies taken from psoriatic lesions of patients shows enrichment of IL-17 producing cells such as Th17 (T-helper 17) cells, cytotoxic T cells and neutrophils, suggesting that excessive infiltration of these cells may be associated with the development of psoriasis(Malakouti et al. 2015). IL-17A is a member of the IL-17 family cytokines, and has been recognized as a key factor in the establishment of psoriatic phenotypes. When IL-17 binds to its receptor present on keratinocytes, dendritic cells and dermal fibroblasts, various pro-inflammatory cytokines and chemokines are released, leading to the development of psoriasis(Jin and Dong 2013). Consequently, various drugs targeting these cytokines have been tested for their anti-inflammatory activities in the context of psoriasis, including anti-TNF agent (etanercept)(Thomson 2007) and anti-IL-17 agent (secukinumab)(Abrouk et al. 2017).

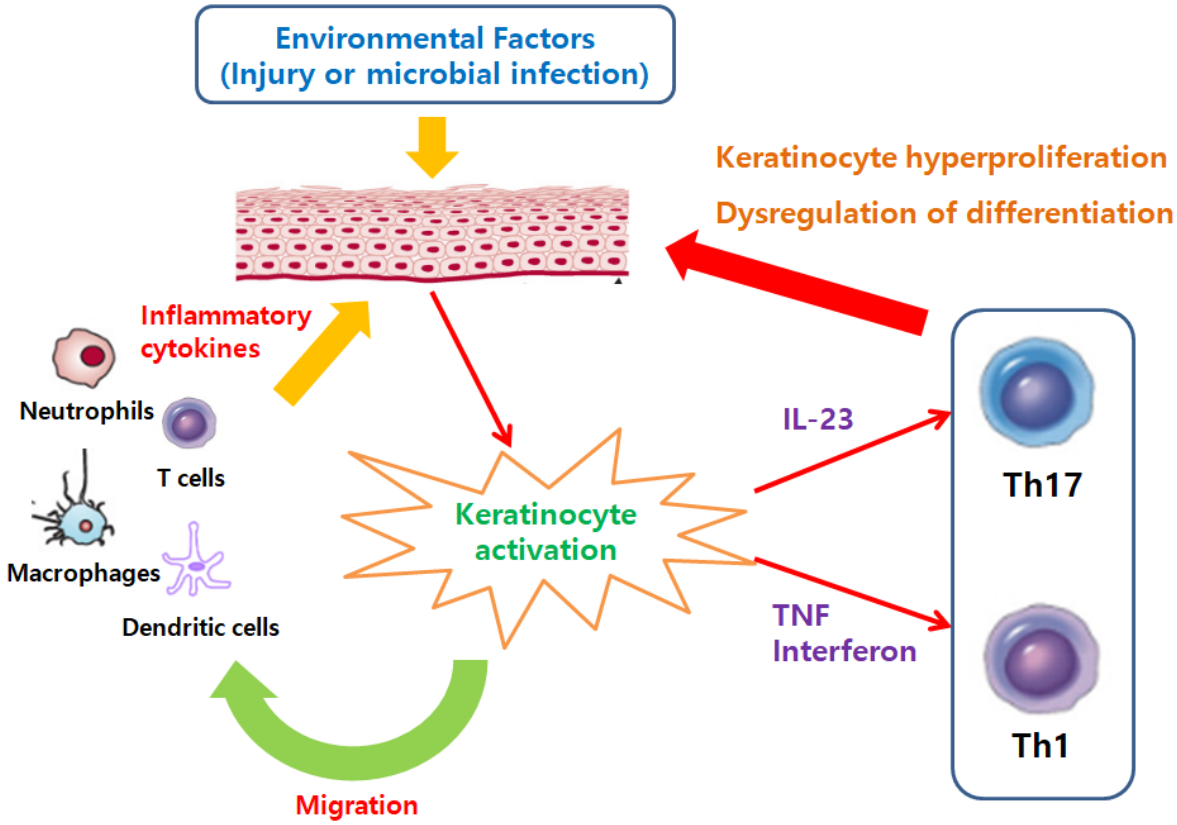


Figure 1. Pathogenesis of psoriasis. Exposure to environmental factors such as microbial or mechanical injury damage leads to activation of antigen presenting cells like macrophages and dermal dendritic cells, followed by keratinocyte activation. Stimulated keratinocytes secrete various inflammatory mediators which promote other inflammatory cells to infiltrate and activate pathogenic T cells. Furthermore, the interaction of antigen presenting cells and T cells leads to the activation of Th1 and Th17 cells mediated by IL-23. Liberation of IL-17 and IL-22 by Th17 cells, and TNF-α and IFN-γ by Th1 cells further perpetuates the keratinocyte injury creating a vicious positive feedback cycle.

During pregnancy, a number of hormonal and immunological changes can alter the severity of pre-existing cutaneous diseases and cause the onset of new cutaneous diseases(Robinson and Klein 2012). Specifically, pregnancy triggers an immune shift from Th1 (T-helper 1) to Th2 (T-helper 2), and also leads to a decrease in Th17 population(Figueiredo and Schumacher 2016). Therefore, it has been predicted that pregnancy is associated with an improvement in numerous Th1 and Th17 immune-mediated diseases including psoriasis. These immunological changes during pregnancy have been reported to be due to increased estrogen levels. In several studies on mice and humans, a significant decrease in the Th1- and Th17-mediated responses occurred during pregnancy or treatment with estrogen(Schumacher, Costa, and Zenclussen 2014). Taken together, estrogen may be important for the development of psoriasis.

Therapeutic approaches : Immunosuppressive drugs such as corticosteroid, vitamin D analogues, cyclosporine and methotrexate are widely used to treat psoriasis, but these drugs have limitations in that they produce not-so-trivial side effects and only temporarily alleviate symptoms(Shutty and Hogan 2013). So-called “rotational” therapy has been proposed to minimize the toxicity of these medicines. The idea is to change the anti-psoriasis drugs every 6 to 24 months in order to minimize the toxicity of one medication(van de Kerkhof 2001). Depending on the medications selected, this method can be used as an option.

An exception to “rotational” therapy is the use of the biologic medications. Biologics are drugs made from complex molecules generating from microorganisms, plants or animal cells. All biologics work by suppressing specific factors involved in the immune inflammatory responses that are overactive in psoriasis(Clements, Abdul-Ghaffar, and Griffiths 2006). Currently, the main classes of biologic drugs for psoriasis are TNF blockers and those that interfere with inflammatory mediators. Currently available biologics for psoriasis include adalimumab (Humira), infliximab (Remicade), etanercept (Enbrel), ustekinumab (Stelara), secukinumab (Cosentyx) and ixekizumab (Taltz)(Clements, Abdul-Ghaffar, and Griffiths 2006).

As with immunosuppressive drugs, all biologic drugs can also produce side effects(Aubin, Carbonnel, and Wendling 2013). Common side effects include mild local injection-site reactions (redness and tenderness). Furthermore, there is the concern of serious infections and potential malignancy with nearly all biologic drugs(Aubin, Carbonnel, and Wendling 2013). These drugs may not be an ideal choice for patients with a history of cancer and those actively undergoing cancer therapy. It was also reported that there may be an increased association of lymphoma in patients taking biologics(Bucher et al. 2005). Therefore, there is a significant unmet medical need for the development of safe and more effective methods for managing psoriasis.

4. Osteoporosis

Osteoporosis is a disease characterized by the decrease in bone density, resulting in fragile bones(Casey 2015). Osteoporosis leads to abnormally porous bone, and results in frequent fractures (breaks)(Diab and Watts 2013). Bones that are affected by osteoporosis can break with relatively minor injury that normally would not cause fracture. The fracture can be either in the form of cracking (as in a hip fracture) or collapsing (as in a compression fracture of the vertebrae). Although osteoporosis-related fractures can occur in almost any skeletal bone, the spine, hips, ribs and wrists are the most common areas of bone fractures from osteoporosis(Jackuliak and Payer 2014). Osteoporosis can be undetected for decades because it does not cause any noticeable symptoms until bone fractures. The symptom most often associated with osteoporotic fractures is pain; the location of the pain depends on the location of the fracture(Mattia et al. 2016).

Osteoporosis affects men and women of all races. However, white and Asian women — especially older women who are post-menopause — are at highest risk(Albala and Pumarino

1996). Currently, more than 200 million people are estimated to be suffering from this disease worldwide, with the number of patients expected to grow rapidly with the rise of the elderly population(Cauley 2017). It has been suggested that approximately one in two women and up to one in four men of age 50 and older will break a bone due to osteoporosis(Albala and Pumarino 1996). The symptoms of osteoporosis in men are similar to those in women. The quality of life in patients with osteoporosis is often diminished because of osteoporotic bone fractures(Madureira, Ciconelli, and Pereira 2012).

Pathophysiology : The underlying pathogenesis of osteoporosis is an imbalance between bone resorption and bone formation(Khosla 2010), for example excessive bone resorption by osteoclasts and inadequate bone formation by osteoblasts. In normal bone, matrix remodeling of bone is constant; up to 10% of all bone mass may be undergoing remodeling at any point in time(Khosla 2010). Osteoclasts regulated by NFATc1 (Nuclear factor of activated T-cells, cytoplasmic 1) degrade the bone matrix(Kim and Kim 2014), while osteoblasts regulated by RUNX2 (Runt-related transcription factor 2) rebuild it(Phimphilai et al. 2006). Low bone mass density can then occur when osteoclasts degrade the bone matrix faster than the rate osteoblasts rebuild it.

Trabecular bone (or cancellous bone) is the sponge-like bone in the ends of long bones and the vertebrae. Cortical bone is the hard outer shell of bones and the middle of long bones. Because osteoblasts and osteoclasts are located on the bone surface, trabecular bone is more exposed to bone turnover and remodeling(Goldstein et al. 1991). Not only the bone density, but also the microarchitecture of bone is disrupted(Legrand et al. 2000). Common osteoporotic fracture sites — the wrist, the hip and the spine — have a relatively high「trabecular bone to cortical bone」ratio, so the intense bone remodeling causes these areas to degenerate most(Iolascon et al. 2013).

Hormones significantly influence the rate of bone resorption; lack of estrogen (e.g. as a result of menopause) facilitates bone resorption and slows down the deposition of new

bone(Figure 2)(Gambacciani and Vacca 2004). The α-form of the estrogen receptor appears to be the most important element in the regulation of bone remodeling(Stewart and Ralston 2000). In addition to estrogen, calcium metabolism plays an important role(s) in bone turnover. Deficiency in both calcium and vitamin D leads to impaired bone deposition. Furthermore, it has been reported that PTH (Parathyroid hormone) secreted by the parathyroid glands increases bone resorption to ensure sufficient calcium in the blood(Morley, Whitfield, and Willick 2001). The role of calcitonin, a hormone generated by the thyroid that increases bone deposition, is less clear, and probably not as significant as that of PTH(Mehta, Malootian, and Gilligan 2003).

The differentiation of osteoclasts is regulated by various molecular signals, of which RANKL (receptor activator of nuclear factor kappa-B ligand) is one of the best studied. This molecule is produced mainly by osteoblasts and stimulates RANK (receptor activator of nuclear factor κB), resulting in the activation of osteoclasts through up-regulation of NF-κB and MAPK (mitogen-activated protein kinase) signaling pathways(Takayanagi et al. 2002). OPG (osteoprotegerin) binds to RANKL before it has an opportunity to bind to RANK, and therefore suppresses its ability to increase bone resorption(Xu et al. 2012). Local production of eicosanoids and interleukins is thought to participate in the bone resorptive activities of osteoclasts, and excess or reduced production of these mediators may influence the development of osteoporosis(Fuller and Chambers 1989).

Osteoblastogenesis, namely the differentiation process of osteoblasts from precursor cells, is regulated by endocrine and paracrine factors such as estrogen and growth factors that activate a variety of intracellular signaling pathways. In osteoblastogenesis, BMP-2 (bone morphogenetic protein-2) plays a master role in the regulation of various genes involved in osteoblast functions such as RUNX2, ALP (alkaline phosphatase), osteocalcin and OPG, by activating the Smad signaling pathway(Phimphilai et al. 2006). Recently, it has been reported that Wnt/β-catenin signaling, which is involved in embryonic developmental processes, also regulates osteoblast differentiation by cross-talking with BMP-2 signaling pathway(Sato et al. 2009).

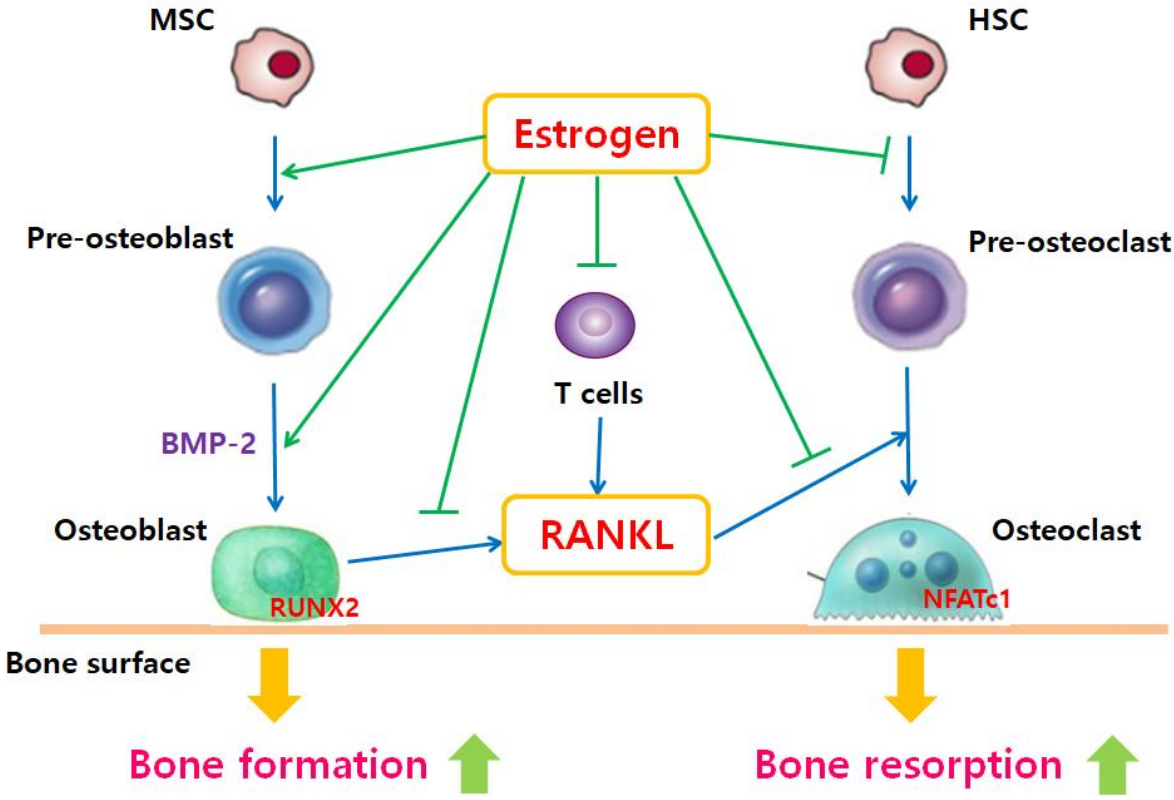


Figure 2. Pathogenesis of osteoporosis. The main mechanisms of osteoporosis development are excessive bone resorption by osteoclasts, and inadequate bone formation by osteoblasts. Estrogen is the most important hormone in the development of osteoporosis. Estrogen enhances bone formation by stimulating osteoblast differentiation and function. Furthermore, it suppresses bone resorption by inhibiting osteoclast differentiation and activity through direct cellular effects and reduced production of RANKL by activated T cells, mesenchymal stem cells (MSCs), osteoblasts and osteocytes.

Recently, growing understanding of the bone remodeling process suggests that factors involved in inflammation are linked with bone physiology and remodeling, supporting the argument that inflammation significantly contributes to the pathogenesis of osteoporosis(Ginaldi, Di Benedetto, and De Martinis 2005). Clinical observations reveal coincidence of systemic osteoporosis with period of systemic inflammation. Furthermore, different epidemiologic studies suggest that the risk of developing osteoporosis is increased in various inflammatory conditions(Mundy 2007). In the bone remodeling processes, pro-inflammatory cytokines such as IL-1β, IL-17 and TNF-α cause an imbalance in bone metabolism by favoring bone resorption via the induction of RANKL and ICAM-1 (intercellular adhesion molecule 1) in osteoblasts(Weitzmann 2013). These inflammatory signals originate from the immune system, the largest source of cell-derived regulatory signals, and such immunological signals to the bone are transmitted primarily via osteoblasts to induce osteoclast differentiation and maturation, resulting in osteoporosis(Bussard, Venzon, and Mastro 2010).

Therapeutic approaches : The goal of treatment of osteoporosis is to prevention bone fractures by reducing bone loss or increasing bone density(Bonnick and Shulman 2006). Although early detection and timely treatment of osteoporosis can substantially decrease the risk of future fractures, none of the available treatments for osteoporosis can produce complete cures. In other words, it is difficult to completely rebuild bone that has been weakened by osteoporosis. Therefore, the prevention of osteoporosis is as important as its treatment. The following are the currently used prevention measures and treatment medications for osteoporosis(Khan 2017; Rosen 2017) :

1. Lifestyle changes including quitting cigarettes, curtailing excessive alcohol intake, exercising regularly and consuming a balanced diet with adequate calcium and vitamin D intake

2. Medications that stop bone loss and increase bone density such as bisphosphonate (alendronate (Fosamax), risedronate (Actonel), ibandronate (Boniva) and zoledronate (Reclast)), calcitonin (Calcimar) and anti-RANKL antibody (denosumab (Prolia))
3. Medications that increase bone formation such as teriparatide (Forteo)

Building strong and healthy bones requires an adequate dietary intake of calcium beginning in childhood and adolescence for both sexes(Sunyecz 2008). However, high dietary calcium intake or taking calcium supplements alone is not sufficient in treating osteoporosis and should not be considered as an alternative to or substituted for more potent prescription medications to treat osteoporosis. For example, in the first several years after menopause, rapid bone loss may occur even when calcium supplements are taken(Aggarwal and Nityanand 2013).

HRT (hormone replacement therapy) involving synthetic 17β-estradiol or conjugated equine estrogens has been commonly used to treat postmenopausal osteoporosis(Gambacciani and Levancini 2014). HRT after menopause has been reported to prevent bone loss, increase bone density, and prevent bone fractures. However, due to the adverse effects of HRT, such as increased risks of heart attack, stroke and breast cancer, HRT is no longer recommended for long-term use in therapies for osteoporosis(Gambacciani and Levancini 2014). Rather, HRT is used for a short term to relieve post-menopausal hot flashes. To overcome the side effects of HRT, SERMs (selective estrogen receptor modulators), such as raloxifene and phytoestrogens, were developed to reap the benefits of estrogen while avoiding its potential side effects(Cranney and Adachi 2005).

Currently, the most effective medications for osteoporosis that have been approved by the FDA are anti-resorptive agents, which inhibit the removal of calcium from bones(Stepan et al. 2003). The bone homeostasis is an essential part of maintaining the normal calcium level in the blood and serves to repair tiny cracks in the bones that occur with normal daily activity(Nakashima 2013). Osteoporosis results when the rate of bone resorption exceeds the rate of bone formation. Anti-resorptive medications inhibit bone resorption, thus controlling the

balance in favor of bone formation, but their long-term use is known to generate side effects such as severe musculoskeletal pain and hypocalcemia(Kennel and Drake 2009). Therefore, there is a significant unmet medical need for the development of alternative treatment methods with fewer side effects for managing osteoporosis.

5. Rationale and purpose of this study

Among previous findings from our laboratory, I paid a special attention to the fact that DHCA contained many, not just a few, potent biological activities. In particular, I was interested in identifying the "master" molecule or the most significant cellular target that DHCA might affect to generate such a diverse range of biological effects. To this end, two approaches were taken; one is in the context of inflammation and the other based on the fact that DHCA is a member of phytoestrogens. Inflammation was chosen because (1) it is one of key pathologic factors in all major human diseases, and (2) DHCA has previously been shown to contain strong anti-inflammatory activities. The idea that DHCA may act as a phytoestrogen was taken because many previous observations could be explained if DHCA interacted with estrogen receptors.

To study the effects of DHCA on inflammation, I initially focused on IL-17-induced inflammation. HaCaT keratinocyte cell line was used because it is the most frequently used cell line model when IL-17 mediated responses are investigated. Cells were treated with IL-17 and DHCA, and its effects were tested on cell proliferation and expression levels of pro-inflammatory cytokines, chemokines and anti-microbial peptides. After observing inhibitory effects of DHCA on those molecules, the well-known mouse model for psoriasis was employed to test whether effects of DHCA observed *in vitro* could be reproduced *in vivo*.

To study whether DHCA could indeed act as a biologically active phytoestrogen, the effects of DHCA were investigated on osteoblastogenesis using MC3T3-E1 and also on osteoclastogenesis employing RAW264.7, which are all gold standard cell lines commonly used in respective studies. DHCA was indeed found to promote the differentiation of osteoblast,

while inhibiting the differentiation of osteoclast. Based on these findings, it was investigated whether DHCA could indeed affect the disease progression in the mouse osteoporosis model. Finally, it was tested whether DHCA could bind to ERα and ERβ. In summary, my thesis research was focused on understanding the molecular mechanisms of DHCA underlying its diverse biological activities in the context of inflammation and its estrogenic behaviors.

Chapter II

Materials and Methods

1. Cell culture and Reagents

1.1 DHCA and other chemical and biological reagents

Synthetic DHCA was produced by previously described methods(Hu and Jeong 2006) and obtained from Biochemnet (Seoul, Korea). IL-17, collagenase type 1, dispase, PMA, Ionomycin, RANKL, L-ascorbic acid, Sodium tartrate dehydrate, 4-nitrophenyl phosphate sodium, 17β-Estradiol, H89, STO609, fulvestrant, BMP-2 and dexamethasone were purchased from Sigma (St Louis, MO, USA). MPP and PHTPP were from R&D systems (Minneapolis, MN, USA).

1.2 HaCaT cell line

HaCaT cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37℃ under 5% CO_2.

1.3 RAW264.7 cell line

RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Minimum Essential Medium Alpha (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37℃ under 5% CO_2.

1.4 MC3T3-E1 cell line

MC3T3-E1 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Minimum Essential Medium Alpha (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37℃ under 5% CO_2.

1.5 Primary skin cell isolation and ex vivo culture

Primary skin cells were isolated using enzymatic digestion method as described previously. Briefly, back skins were treated with DMEM in the presence of collagenase type 1 (1mg/mL) and dispase (1mg/mL) for 30 min. Skin cells were then physically isolated through mincing with a 40μm nylon cell strainer (BD bioscience, Franklin Lakes, NJ, USA), and were subsequently cultured in DMEM in the presence of PMA (20ng/mL) and Ionomycin (500ng/mL) for 3 days. Supernatants were collected followed by IL-17 specific ELISA (R&D systems, Minneapolis, MN, USA).

1.6 Osteoclast differentiation in vitro

For osteoclast differentiation experiments, RAW264.7 cells were plated at 3×10^3 cells per well in 96-well culture plates containing α-MEM with 10% FBS. Twenty-four hours later, cells were treated with 50ng/mL of RANKL and various concentrations of DHCA. After 5 days in culture, the cells were subjected to Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions.

1.7 Osteoblast differentiation in vitro

For the osteoblast differentiation experiments, MC3T3-E1 cells were plated at 2×10^3 cells per well in 96-well culture plates containing α-MEM with 10% FBS. Twenty-four hours later, cells were treated with 25ng/mL of BMP-2 and various concentrations of DHCA. After 5 days in culture, the cells were subjected to Leukocyte Alkaline Phosphatase (ALP) Kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions.

2. Molecular cellular biological techniques

2.1 Measurement of pro-inflammatory cytokines

The production of TNF-α, IL-6 and IL-1β in culture supernatant was evaluated by ELISA. Briefly, HaCaT cells were treated with IL-17 (100ng/mL) and various concentrations of DHCA. Twenty-four hours later, the culture supernatant was collected and the amount of cytokines was measured by specific ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

2.2 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

To determine the RNA level in HaCaT cells, HaCaT cells were treated with IL-17 (100ng/mL) and various concentrations of DHCA for 24 h, and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using an oligo dT primer (Qiagen, Valencia, CA) and AMV reverse transcriptase (TaKaRa, Shiga, Japan). One microliter of this cDNA per sample was used for quantitative polymerase chain reaction using SYBR Premix Ex TaqTM (TaKaRa, Shiga, Japan).

Conditions for PCR were denaturation at 95℃ for 5 seconds, annealing and extension at 60℃ for 20 seconds.

To determine the RNA level in RAW264.7 cells, cells were treated with 50ng/mL of RANKL and various concentrations of DHCA. After 24 hours in osteoclast differentiation, total RNA was isolated using TRIzol reagent according to the manufacturer's protocol. The identical qRT-PCR procedures were performed as used to detect the RNA level of HaCaT cells.

To determine the RNA level in MC3T3-E1 cells, cells were treated with 25ng/mL of BMP-2 and various concentrations of DHCA. After 24 hours in osteoblast differentiation, total RNA was isolated using TRIzol reagent according to the manufacturer's protocol. The identical qRT-PCR procedures were performed as used to detect the RNA level of HaCaT cells. The primer sequences used in this study were described in Table 2.

2.3 Western blot analysis

To determine the protein level in HaCaT cells, cells were plated in 100mm culture dishes. Twenty-four hours later, cells were treated with IL-17 (100ng/mL) and various concentrations of DHCA for 30 min. After treatment, cells were washed with cold PBS and lysed with phosphosafe extraction buffer (Novagen, Madison,WI, USA). Total proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were incubated with primary antibodies for 16 hours, then were treated with horse radish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:100,000, Sigma) and visualized in films using ECL solution (Millipore, Billerica, MA).

To determine the protein level in RAW264.7 cells, cells were plated in 100mm culture dishes. Twenty-four hours later, cells were treated with RANKL (50ng/mL) and various concentrations of DHCA for 30 min The identical Western blot procedures were performed to detect the protein level of HaCaT cells.

To determine the protein level in MC3T3-E1 cells, cells were plated in 100mm culture dishes. Twenty-four hours later, cells were treated with BMP-2 (25ng/mL) and various

Primers	Forward	Reverse
hTNF-α	CAAAGTAGACCTGCCCAGAC	GACCTCTCTCTAATCAGCCC
hIL-6	CGGGAACGAAAGAGAAGCTCTA	CGCTTGTGGAGAAGGAGTTCA
hIL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
hCXCL1	AATCCTGCATCCCCCATA	TGTCTCTCTTTCCTCTTCTGTTCCT
hCXCL8	TTGGCAGCCTTCCTGATTTC	TATGCACTGACATCTAAGTTCTTTAGCA
hCCL20	CTGGCTGCTTTGATGTCAGT	CGTGTGAAGCCCACAATAAA
hS100A8	GGGAATTTCCATGCCGTCT	CCTTTTTCCTGATATACTGAGGAC
hS100A9	CAGCTGGAACGCAACATAGA	TCAGCTGCTTGTCTGCATTT
hLL-37	TCGGATGCTAACCTCTACCG	GGGTACAAGATTCCGCAAAA
mIL-17A	ATCCCTCAAAGCTCAGCGTGTC	GGGTCTTCATTGCGGTGGAGAG
mIL-17F	ACCCGTGAAACAGCCATGGTCAAG	CCCATGGGGAACTGGAGCGG
mIL-22	TTTCCTGACCAAACTCAGCA	CTGGATGTTCTGGTCGTCAC
mTNF-α	ACTGAACTTCGGGGTGATCGGTCC	GTGGGTGAGGAGCACGTAGTCG
mIL-6	GCCTTCTTGGGACTGATG	CTGGCTTTGTCTTTCTTGTTA
mIL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACTG
mS100A8	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA
mS100A9	ATACTCTAGGAAGGAAGGACACC	TCCATGATGTCATTTATGAGGGC
mLL-37	GATAACAAGAGATTTGCCCTGCTG	TTTCTCAGAGCCCAGAAGCCTG
mTRAP	GGTCAGCAGCTCCCTAGAAG	GGAGTGGGAGCCATATGATTT
mC-Fos	ACTTCTTGTTTCCGGC	AGCTTCAGGGTAGGTG
mNFATc1	GGAGAGTCCGAGAATCGAGAT	TTGCAGCTAGGAAGTACGTCT
mMMP-9	GGACCCGAAGCGGACATTG	GAAGGGATACCCGTCTCCGT
mDC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
mCathepsin K	AGGCAGCTAAATGCAGAGGGTACA	ATGCCGCAGGCGTTGTTCTTATTC
mALP	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
mOsteocalcin	CTGACCTCACAGATGCCAAG	GTAGCGCCGGAGTCTGTTC
mOPG	GTTTCCCGAAGGACCACAAT	CCATTCAATGATGTCCAGGAG

Table 2. The primer sequences used in qRT-PCR analysis

concentrations of DHCA for 30 min. The identical Western blot procedures were performed to detect the protein level of HaCaT cells. The primary antibodies used in this study were as follows: p-p38 (1:500, Cell Signaling, Beverly, MA), p38 (1:1000, Cell Signaling), p-ERK (1:500, Cell Signaling), ERK (1:1000, Cell Signaling), p-JNK (1:1000, Cell Signaling), JNK (1:1000, Cell Signaling), p-Akt (1:500, Cell Signaling, Beverly, MA), Akt (1:1000, Cell Signaling), p-IKKβ (1:500, Santa Cruz Biotechnology, SantaCruz, CA), IKKβ (1:500, Santa Cruz), IκB (1:500, Santa Cruz), p-AMPKα1 (1:1000, Cell Signaling), AMPKα1 (1:1000, Cell Signaling), RUNX2 (1:1000, Cell Signaling, Beverly, MA), p-Smad1/5/9 (1:1000, Cell Signaling), Smad1/5/9 (1:1000, Abcam, Cambridge, MA, USA) and β-actin (1:5000, Sigma).

2.4 Measuring TRAP activity

After osteoclast differentiation, TRAP activities of osteoclasts were measured in the well by incubating them for 15–30 min at 37°C with 30μl of 600 mM sodium acetate buffer (pH 5.5) containing L-ascorbic acid (17.6 mg/mL), sodium tartrate dehydrate (9.2 mg/mL), 4-nitrophenylphosphate Na (3.6 mg/ml), Triton X-100 (0.3%), EDTA (6 mM), and NaCl (600 mM). The reaction was terminated by the addition of 30μl of NaOH (300 mM), and activities were measured at 405 nm.

2.5 Measuring ALP activity

After osteoblast differentiation, ALP activities of osteoblasts were measured in the well by incubation for 30 min at 37℃ with 100μl of Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System for ELISA (Sigma, St Louis, MO, USA) containing 1% Tween-20. The reaction was terminated by the addition of 50μl of NaOH (300 mM), and activities were measured at 405 nm.

2.6 siRNA transfection

The siRNA specific for AMPKα1, ERα, ERβ and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA was transfected into cells using the RNAiMAX (ThermoFisher Scientific, Woburn, MA, USA) according to the manufacturer's protocol. After 48h, the cells were subjected to the analysis. Knock-down efficiency was evaluated using primary antibodies against AMPKα1 (1:1000, Cell Signaling), ERα (1:1000, Cell Signaling) and ERβ (1:1000, Cell Signaling).

2.7 Luciferase reporter plasmid assay

Inducible estrogen responsive element (ERE)-responsive luciferase reporter plasmid was purchased from QIAGEN (Valencia, CA). Luciferase reporter plasmid assay was performed as described previously(Lee, Choi, and Kim 2015). Briefly, cells were transiently transfected with ERE-reporter plasmid and a β-galactosidase plasmid (1μg, Invitrogen), using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with 17β-estradiol (10nM) and various concentrations of DHCA for 24h. Cell lysates were prepared, and a luciferase activity assay was performed using the Luciferase Reporter kit according to the manufacturer's protocol (Promega, Madison, WI, USA) with a microplate luminometer (MicroLumat Plus LB96V, Berthold, Germany). Luciferase activity was normalized to β-gal activity.

2.8 Sequence alignment and homology modeling

The amino acid sequences of ERα and ERβ for *Mus musculus* (mERα and mERβ, accession number: BAJ65337 and AAB51132) and *Homo sapiens* (hERα and hERβ, accession number: 2OCF_A and 2J7X_A) were retrieved from the NCBI protein sequence database.

Global pairwise sequence alignment of mERα and mERβ amino acid sequences was performed using the EMBOSS package to calculate sequence identity and similarity among the species. To identify a suitable template, the amino acid sequences of mERα and mERβ were searched against the Protein Data Bank (PDB) database using the PSI-BLAST algorithm. The crystal structures of hERα for mERα and hERβ for mERβ (PDB ID: 2OCF and 2J7X) were used as templates to build a mERα and mERβ structure. Homology modeling of the mERα and mERβ was performed using the Prime homology modeling program of Schrödinger. The crystallographic positions of the backbone atoms and conserved side chains were mapped from the template, while the side chain coordinates of all non-identical residues were predicted.

2.9 Molecular docking simulation

Ligand-receptor molecular docking was simulated by Glide (Schrödinger). Grid-generation module from Glide was used to generate grids for the mERα and mERβ structures produced through homology modeling. The scaling factor of the Van der Waals radii was set to 0.8 and the partial charge cutoff to 0.15. The binding site of mERα or mERβ was included in the grid generation. Dehydrodiconiferyl alcohol and estradiol, the selected ligands, were drawn by 2D sketcher and optimized with MacroModel. All possible ionization states and stereoisomer structures of the ligands were generated using the Ionizer option in LigPrep. Five poses per ligand, while performing the docking of these two compounds to mERα and mERβ, were produced by the SP mode of Glide, respectively. The ligand interaction diagram module of Glide was used to analyze ligand-protein interactions.

2.10 Calculation of binding energy

The Prime molecular mechanics based generalized born/surface area (MM-GBSA) model of the Schrödinger suite was used to calculate the free energy of binding of the ER-ligand

complex from the docking simulations. The binding free energy (ΔG_{bind}) was evaluated as:

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G_{solv} + \Delta G_{SA}$$

where ΔE_{MM} is the difference in the minimized energies between the ER-ligand complex and the sum of the energies of the free ER and ligand. ΔG_{solv} is the difference between the GBSA solvation energy of the ER-ligand complex and the sum of the solvation energies of free ER and ligand. ΔG_{SA} is the difference between the surface area energies of the complex and the sum of the surface area energies of free ER and ligand.

2.11 Estrogen receptor competition assay

Estrogen receptor competition assay was performed using PolarScreen^{TM} ERα / β competitor assay kit (ThermoFisher Scientific, Woburn, MA, USA) according to the manufacturer's protocol. Briefly, prepare a 2X solution of estrogen receptor / Fluormone tracer complex in a 384-well polypropylene black microplates (Corning, NY, USA), and add a test compounds immediately using multi-channel pipet then incubate for 2 hours. Fluorescence polarization values were measured using FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California, USA).

2.12 Statistical analysis

All quantitative data were presented as means ± S.E.M. from three independent experiments. Differences between two groups were statistically analyzed using Student's t-test, whereas one-way ANOVA was used for multiple comparisons. P-values were calculated and when less than 0.05 were considered to be statistically significant.

3. Mouse disease models

3.1 Experimental Animals

All animal protocols were performed in compliance with the guidelines set by the Institutional Animal Care and Use Committee of Seoul National University. Male 7-week-old Balb/c and Female 8-week-old Balb/c mice were purchased from Orientbio Inc. (Seongnam, Korea) and housed in an air-conditioned facility at Seoul National University with a fixed 12 h light/dark cycle.

All animal experiments were carried out in accordance with the Guide for Animal Experimentation of Seoul National University. The protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University.

3.2 IMQ-induced psoriasis-like skin inflammation mice model

IMQ-induced psoriasis-like skin inflammation was generated as described previously. Briefly, 7-wk male Balb/c mice were topically administered followed by applying DHCA (100, 200 and 400 mg/kg) or dexamethasone (10mg/kg) in the presence of 62.5 mg of IMQ, on a daily basis, to the back skin of mice for 5 consecutive days. At the completion of the study, animals were autopsied and serum and back skin were collected followed by CXCL1-, CXCL8- and CCL20- specific ELISA (R&D systems, Minneapolis, MN, USA), H&E staining, immunohistochemistry and quantitative RT-PCR.

3.3 Scoring severity of skin inflammation

To score the severity of the inflammation of the back skin, an objective scoring system was used as described previously. Briefly, erythema, scaling, and thickness were scored daily

from 0 to 4 as follows: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The thickness of back skin was measured using a micrometer (Mitutoyo). The cumulative score (erythema plus scaling plus thickening) served to indicate the severity of inflammation (0–12).

3.4 Histology and Immunohistochemistry

For H&E staining, the back skin was fixed with 10% neutral buffered formalin. Fixed tissues were paraffin-embedded, sectioned and stained with H&E. For immunohistochemistry, sectioned slices were subjected to immunohistochemical staining with antibodies against Ly-6G (Abcam, Cambridge, UK) and CD3 (Abcam) according to the manufacturer's protocol.

3.5 Ovariectomized-mice model

Ovariectomy surgery was performed as described previously(Tyagi et al. 2012). Briefly, all mice were housed and given one week to adapt to their surroundings before surgery. The experimental groups were divided into five groups (sham surgery with vehicle, ovariectomy with vehicle and ovariectomy with various concentrations of DHCA; n=10 mice per group). Sham mice underwent bilateral laparotomy but the ovaries were left in place, while mice in the other four groups underwent bilateral ovariectomy via the ventral approach. After a week of recuperation, all treatments were given by intra-peritoneal route and continued for 4 weeks. At the completion of the study, animals were autopsied and serum samples and femur bones were collected. Serum samples were subjected to NTx (Cusabio, Wuhan, China) and CTx (Cusabio) ELISA according to the manufacturer's protocol. Bone marrow was flushed out from femur bones, total RNA from bone marrow was isolated using TRIzol reagent (Invitrogen) and quantitative RT-PCR was performed. Bones were kept in formalin solution for μCT study. 3D- and 2D- trabecular structure, parameters like bone volume over total volume (BV/TV, %), bone surface density (BS/BV, 1/mm), trabecular thickness (Tb.Th, mm), trabecular spacing (Tb.Sp,

mm), trabecular number (TB.N, 1/mm), trabecular pattern factor (Tb.Pf, 1/mm), structure model index (SMI) and bone mineral density (BMD, g/cm^3) were calculated.

Chapter III

Suppressive Effects of DHCA on IL-17 mediated Inflammatory Responses using Psoriasis as a Model Disease

1. Background

Psoriasis is a chronic inflammatory immune disease characterized by dysregulated keratinocyte proliferation and differentiation, marked infiltration of neutrophil and T cells into the skin, and release of pro-inflammatory cytokines(Lowes, Suarez-Farinas, and Krueger 2014; Valdimarsson et al. 1986). Histological features of psoriatic lesions include a thickened or acanthotic epidermis from epidermal hyperplasia, aberrant keratinocyte differentiation as manifested by the loss of the granular layer, and increased dermal vascularity(Watson et al. 2007). Currently, more than 2-3% of the worldwide population is estimated to be suffering from psoriasis(Michalek, Loring, and John 2017). Many immunosuppressive drugs such as corticosteroid, vitamin D analogues and methotrexate are widely used to treat psoriasis, but these drugs have limitations in that they produce significant side effects while only providing temporary relief of symptoms(Shutty and Hogan 2013). Therefore, there is a significant unmet medical need for the development of safe and more effective methods for managing psoriasis.

Although the pathogenesis of psoriasis is not fully understood, several studies have suggested that IL-17 producing T cells such as Th17 cells and γδ T cells, play an important role(s)(Malakouti et al. 2015). IL-17A, a member of the IL-17 family of cytokines, has been recognized as a key factor in the establishment of psoriatic phenotypes. When IL-17 binds to its receptor present on keratinocytes, dendritic cells and dermal fibroblasts, it leads to the release of various pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-17A and IL-22 and chemokines like CXCL1 (chemokine (C-X-C motif) ligand 1), CXCL8 (chemokine (C-X-C motif) ligand 8 and CCL20 (chemokine (C-C motif) ligand 20), all of which are involved in the development and progression of psoriasis(Jin and Dong 2013; Ha et al. 2014). For example, pro-inflammatory cytokines secreted by T cells and keratinocytes generate inflammatory responses, dysregulated proliferation and differentiation of keratinocytes, and overproduction of antimicrobial peptides from keratinocytes(Wu et al. 2015). Consequently, various drugs targeting these cytokines have been tested for their anti-inflammatory activities in the context of psoriasis, for example, a small molecule against TNF (etanercept) or an antibody to IL-17 (secukinumab) have been tested for their possible use in treating psoriasis(Thomson 2007;

Abrouk et al. 2017).

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from water-soluble extracts of *Cucurbita moschata*(Lee et al. 2012). DHCA was originally found to contain anti-adipogenic(Lee et al. 2012), anti-inflammatory(Lee, Choi, and Kim 2015) and anti-oxidative stress activities(Lee and Kim 2014). More recently, DHCA has been shown to inhibit Th17 cell differentiation by regulating NF-κB activity(Lee et al. 2015). In this study, it was investigated whether synthetic DHCA could suppress IL-17-induced inflammatory responses using human keratinocyte cell line HaCaT. Our data suggested that DHCA inhibited the IL-17-mediated cell proliferation and induction of TNF-α, IL-6 and IL-1β. This lignan molecule suppressed the IL-17-mediated activation of p38 MAPK signaling pathway, downregulating the expression of genes such as pro-inflammatory cytokines, chemokines and antimicrobial peptides. Consistent with these in vitro data, DHCA ameliorated the severity of the clinical scores of psoriasis and the production of pro-inflammatory cytokines in a mouse model of imiquimod-induced psoriasis-like skin inflammation. Data from ex vivo culture experiments showed that treatment with DHCA might reduce the population of IL-17 producing cells in both skin and lymph nodes. Taken together, these data suggested that DHCA might be developed as an efficient therapeutic for psoriasis.

2. Results

2.1 DHCA inhibits IL-17-induced inflammatory responses.

IL-17, a pro-inflammatory cytokine produced mainly by Th17 cells, is thought to induce the hyperproliferation of keratinocytes, which is the major characteristic symptom of psoriasis(Ma, Jia, and Zhang 2016). To test the effects of DHCA on IL-17-mediated increases in cell proliferation, HaCaT cells were treated with IL-17 (100ng/mL) and DHCA (40μM), and cell viability was examined by MTT assay. As shown in Figure 3A, IL-17 promoted cell

proliferation by 2 fold at 72 hours, while DHCA treatment almost completely abolished such an effect. To be certain, the effect of DHCA on the viability of HaCaT cells was measured by the MTT assay at three different concentrations of DHCA (10, 20 and 40 μM). As shown in Figure 3B, DHCA had little effect on cell viability at all concentrations used in this study.

Keratinocytes produce various pro-inflammatory cytokines when stimulated by IL-17(Cho et al. 2012). To test the effects of DHCA on the production of pro-inflammatory cytokines, HaCaT cells were cultured with IL-17 and DHCA (10, 20 and 40 μM) for 24 hours, and the level of TNF-α, IL-6, and IL-1β in the culture supernatants was measured by ELISA. DHCA treatment suppressed the IL-17-mediated increase in the protein level of TNF-α, IL-6, and IL-1β in a dose-dependent manner (Figure 3C). These data suggested that DHCA might inhibit the IL-17-induced inflammatory responses, such as increased cell proliferation and overproduction of inflammatory cytokines in keratinocytes.

2.2 DHCA inhibits the expression of inflammation-related genes.

In keratinocytes, IL-17 activates the production of various pro-inflammatory cytokines, anti-microbial peptides and chemokines, leading to the onset of a variety of immune responses in psoriatic plaques(Johnston et al. 2013). To test the effects of DHCA on these genes, HaCaT cells were treated with IL-17 and DHCA (10, 20 and 40 μM) for 24 hours, and the RNA level was measured by quantitative RT-PCR using specific primers for TNF-α, IL-6, IL-1β, CXCL1, CXCL8, CCL20, S100A8, S100A9 and LL-37. In all cases, IL-17 treatment highly increased their RNA levels by 3-20 fold depending on the genes, while co-treatment with DHCA effectively decreased their levels in a dose-dependent manner (Figure 4).

2.3 DHCA suppresses IL-17-induced p38 MAPK activation.

It has been reported that IL-17 stimulation leads to the activation of MAPK signaling pathways, which is a critical step in the development of inflammatory responses

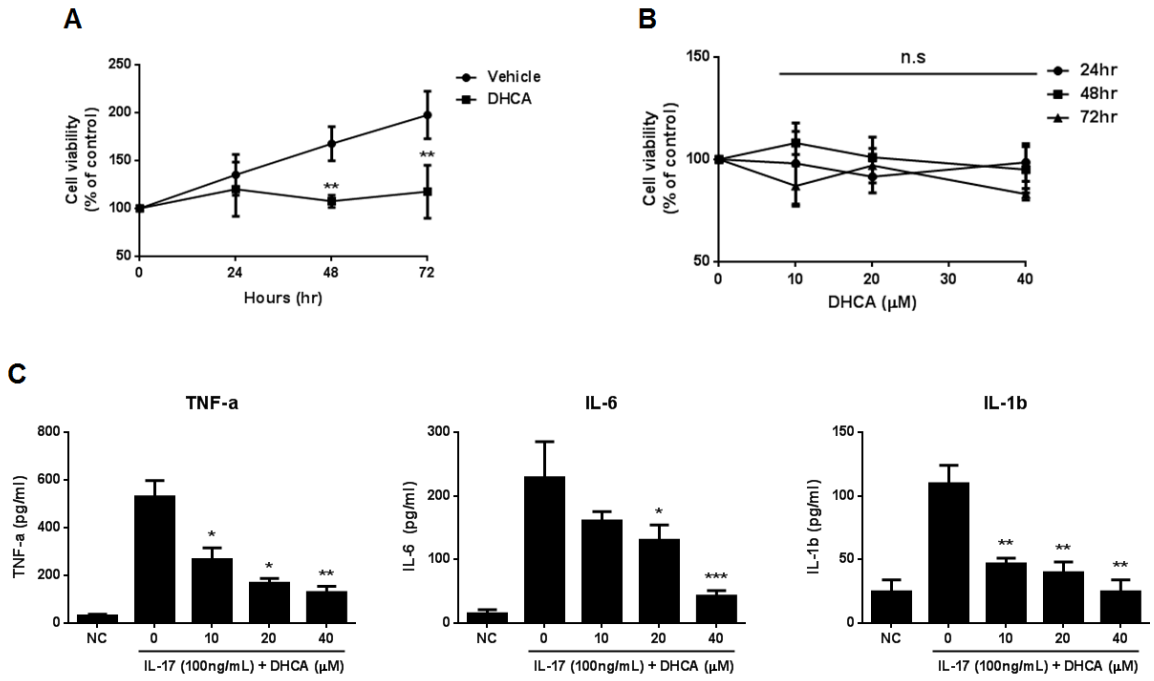


Figure 3. Effects of DHCA on IL-17-mediated inflammatory responses in HaCaT cells. (A) Effect of DHCA on IL-17-induced cell proliferation. HaCaT cells were treated with IL-17 (100ng/mL) and DHCA (40μM) for 72 hours. Cells were then subjected to MTT assay as described in the Materials and Methods section. (B) Effect of DHCA on cell viability. HaCaT cells were treated with three different concentrations of DHCA (10, 20 and 40 μM) for 72 hours. Cells were then subjected to MTT assay as described in the Materials and Methods section. (C) Effects of DHCA on inflammatory cytokines. HaCaT cells were treated with IL-17 (100ng/mL) and DHCA (10, 20 and 40 μM). After 24 hours, culture supernatants were subjected to ELISA for TNF-α, IL-6, and IL-1β. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with that from cells treated with IL-17 alone.

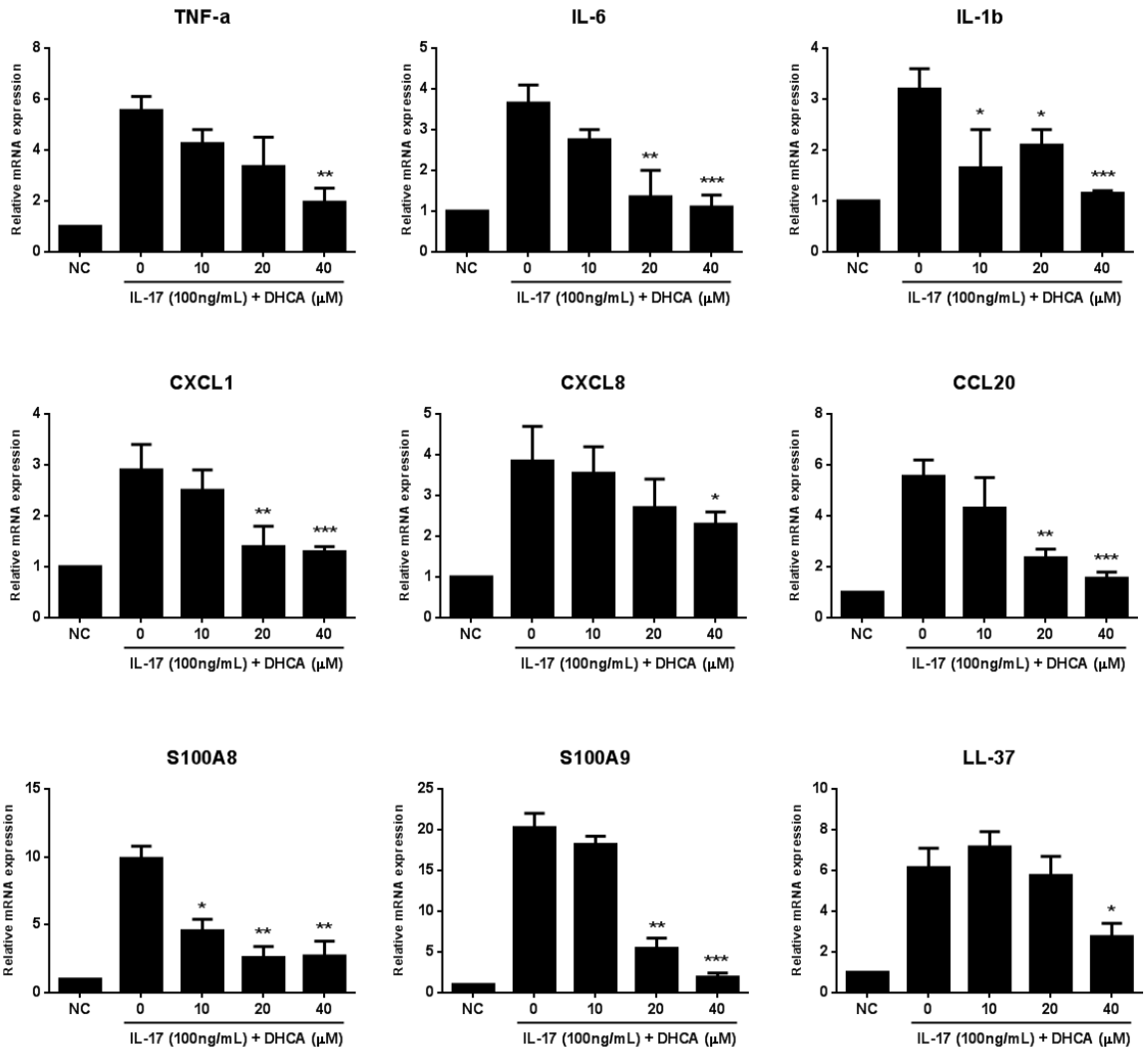


Figure 4. Effects of DHCA on the expression of inflammation-related genes induced by IL-17 in HaCaT cells. HaCaT cells were treated with IL-17 (100ng/mL) and cultured in the presence of DHCA (10, 20 and 40 μM) for 24 hours. Total RNAs were isolated and analyzed by quantitative RT-PCR for TNF-α, IL-6, IL-1β, CXCL1, CXCL8, CCL20, S100A8, S100A9 and LL-37. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with that from cells treated with IL-17 alone.

(Iyoda et al. 2010; Li et al. 2016). To test the effects of DHCA on the IL-17-induced MAPK signaling pathways, HaCaT cells were co-treated with IL-17 (100 ng/mL) and three different concentrations of DHCA (10, 20 and 40 μM) for 30 minutes, and the phosphorylation status of p38 (p38 mitogen-activated protein kinases), ERK (extracellular signal–regulated kinases) and JNK (c-Jun N-terminal kinases) was determined by Western blot. IL-17 stimulation increased phosphorylation of p38, ERK and JNK, and DHCA treatment inhibited p38 phosphorylation in a dose-dependent manner (Figure 5). However, DHCA did not have any effect on the level of phosphorylated ERK and JNK (Figure 5). These data indicated that DHCA negatively controlled the IL-17-induced p38 MAPK signaling pathway.

2.4 DHCA attenuates IMQ-induced psoriasis-like skin inflammation.

The above data suggested that DHCA might produce anti-psoriatic activities by suppressing IL-17-mediated inflammatory responses. To test this possibility in vivo, an IMQ-induced psoriasis-like skin inflammation mouse model was used(van der Fits et al. 2009). Three different concentrations of DHCA (100, 200, 400 mg/kg) and dexamethasone (10 mg/kg), used as positive controls, were topically administered on a daily basis followed by the application of 62.5 mg of IMQ to the back skin of mice for 5 consecutive days. As shown in Figure 6A and B, the back skin of mice treated with IMQ clearly showed psoriatic symptoms such as erythema, scaling and thickening, and this effect was diminished upon DHCA treatment in a dose-dependent manner. It was interesting to note that these ameliorating effects of 400mg/kg DHCA were more effective than those of dexamethasone (Figure 6B). Results from the histological analysis of the IMQ-treated back skin showed inflammatory skin phenotypes including hyperkeratosis and elongation of rete-like ridge(Zhang et al. 2016). When treated with DHCA, however, all of these pathologic features were markedly improved (Figure 6C). Taken together, these data indicated that DHCA might inhibit IMQ-induced psoriasis-like skin inflammation in mice.

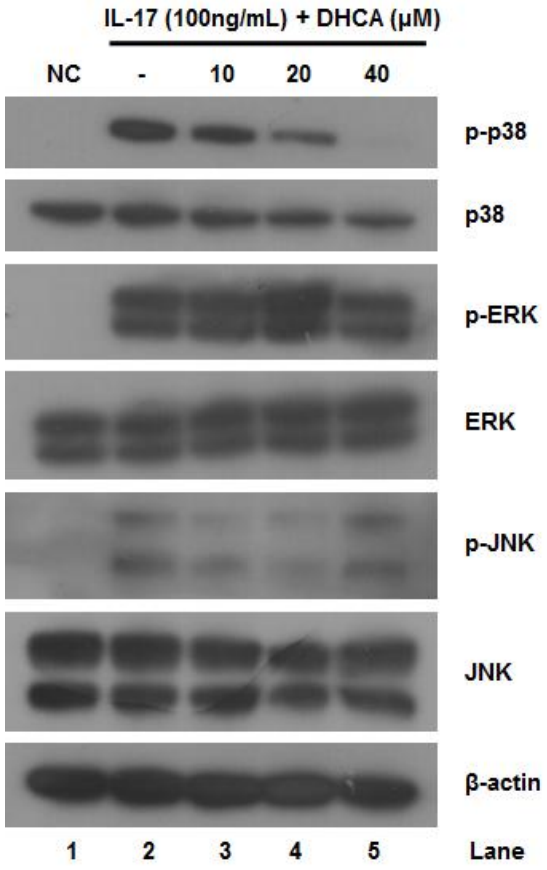


Figure 5. Effects of DHCA on MAPK signaling pathways. HaCaT cells were treated with IL-17 (100ng/mL), and cultured in the presence of DHCA (10, 20 and 40 μM) for 30 min. Total protein were prepared following by western blot using specific antibodies to phosphorylated or unphosphorylated forms of the MAPKs including p38, ERK and JNK.

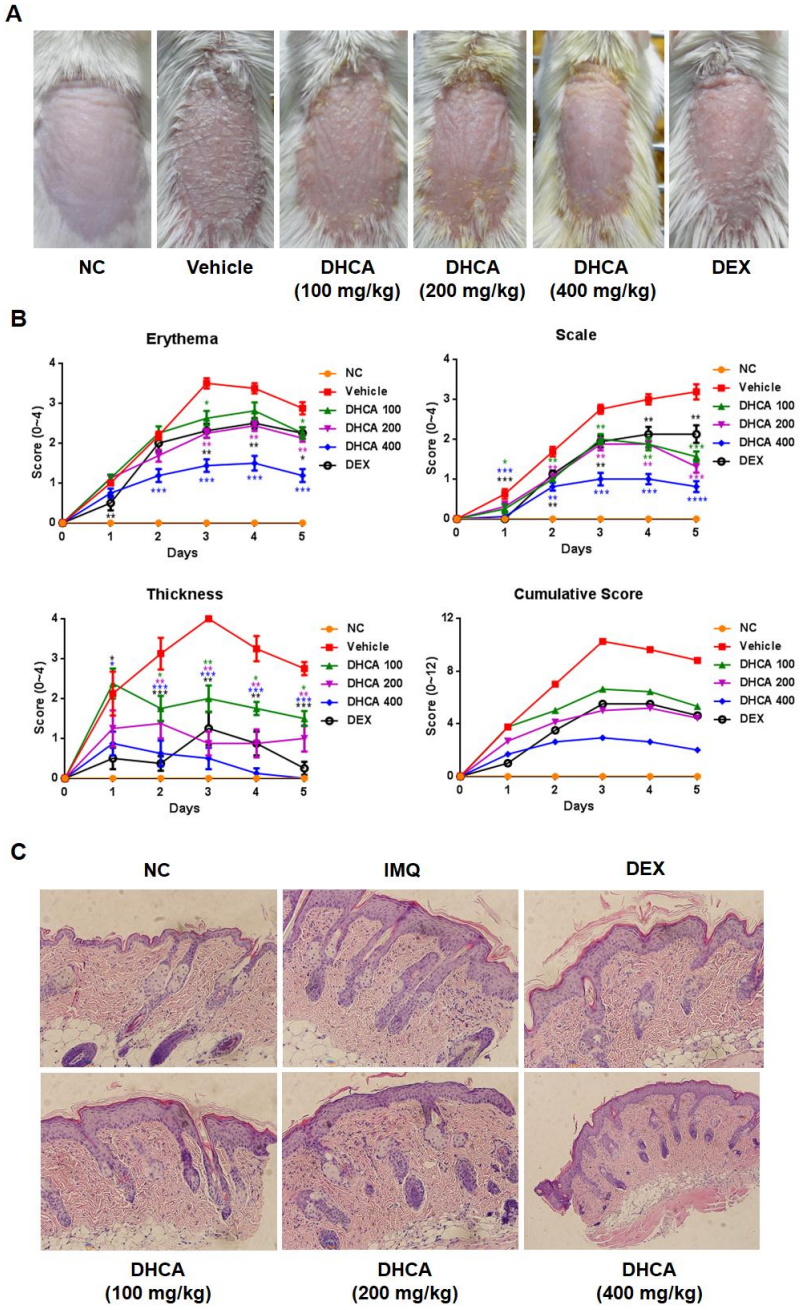


Figure 6. Effects of DHCA on IMQ-induced psoriasis-like skin inflammation in mice. 7-wk male Balb/c mice were topically administered followed by applying DHCA (100, 200 and 400 mg/kg) or dexamethasone (10mg/kg) in the presence of 62.5 mg of IMQ, on a daily basis, to the back skin of mice for 5 consecutive days. (A) Phenotypical presentation of mouse back skin on day 5; (B) Effects of DHCA on disease progression. Erythema, scaling and thickness of back skin were scored daily on a scale from 0 to 4. Additionally, the cumulative score is depicted; (C) Effects of DHCA on histological characteristics in the back skin. H&E staining of the mouse back skin was performed as described in Materials and Methods section. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ compared with Vehicle group.

2.5 DHCA inhibits the expression of psoriasis-associated molecules in psoriatic lesions.

Topical IMQ application activates the expression of psoriasis-associated molecules such as Th17-related cytokines, pro-inflammatory cytokines and anti-microbial peptides in psoriatic skin(van der Fits et al. 2009). To test the effects of DHCA on these genes, mice were treated with IMQ and DHCA (100, 200, 400 mg/kg), and sacrificed at day 5 followed by quantitative RT-PCR analysis to measure the RNA level of various genes such as IL-17A, IL-17F, IL-22, TNF-α, IL-6, IL-1β, S100A8, S100A9 and LL-37 in the back skin. The RNA level of all these genes was highly increased in the IMQ-treated group, while it was reduced by DHCA treatment in a dose-dependent manner (Figure 7).

2.6 DHCA reduces the infiltrations of neutrophils and IL-17-producing lymphocytes in psoriatic lesions.

Recruitment of inflammatory cells such as neutrophils and T cells into the skin is a critical process in the pathogenesis of psoriasis(Prinz 2003; Schon, Broekaert, and Erpenbeck 2017). To test the effects of DHCA on the infiltration of inflammatory cells, mice were treated with IMQ and DHCA, and sacrificed at day 5 followed by immunohistological analysis using antibodies against Ly-6G and CD3 as a marker for neutrophils and T cells, respectively. As shown in Figure 8A, the back skin of mice treated with IMQ contained significant numbers of neutrophils and T cells, but treatment with DHCA lowered their infiltrations in a dose-dependent manner.

It has been reported that IL-17 producing cells are abundant in psoriatic lesions, and may play a key role(s) in the development of psoriasis(Keijsers et al. 2014). To test the effects of DHCA on the population of IL-17-producing cells, primary skin cells were isolated from back skin at day 5, using the enzymatic digestion method, followed by stimulation with

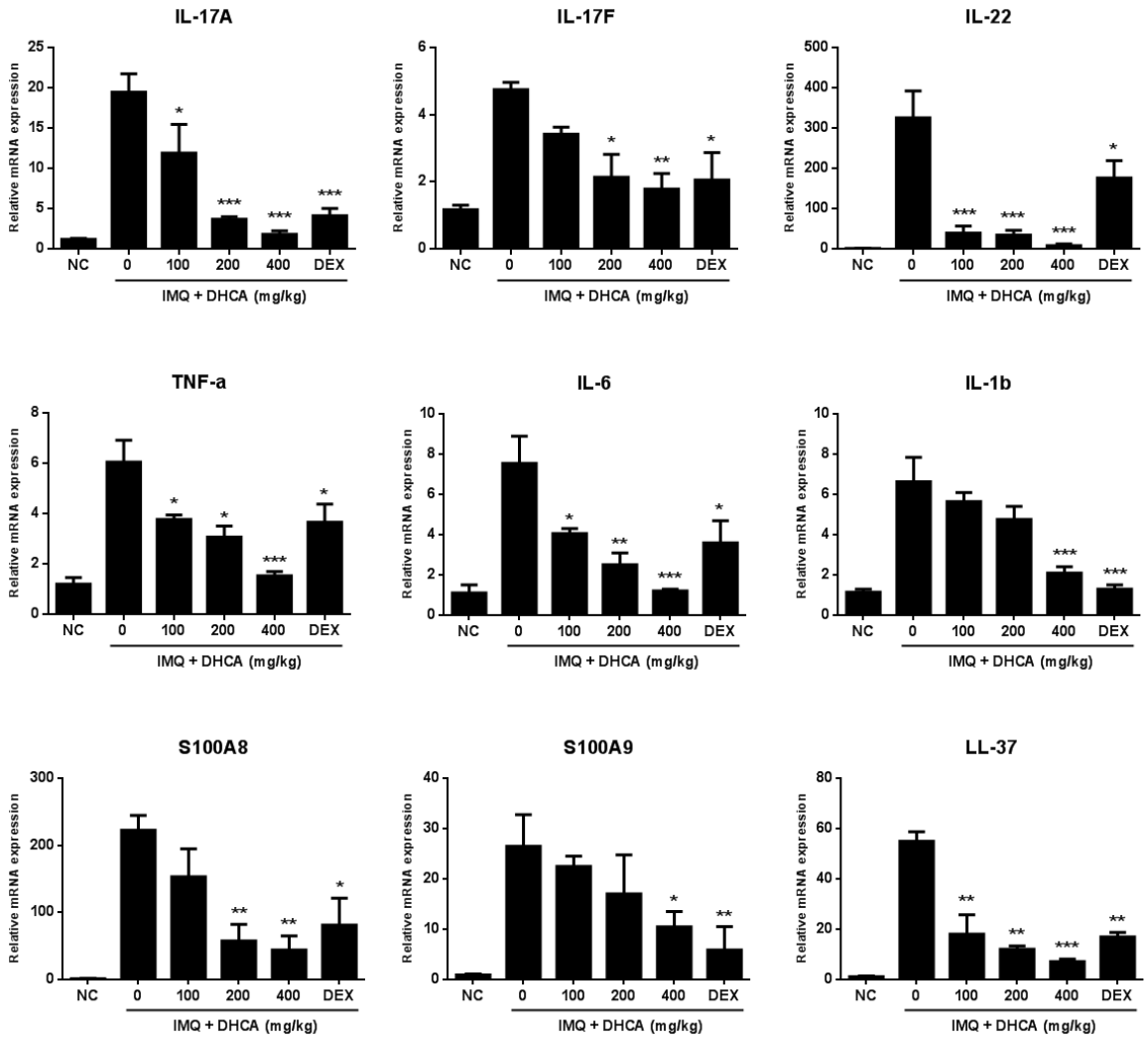


Figure 7. Effects of DHCA on the expression of psoriasis-associated genes in psoriatic lesions. Mice were treated with IMQ in the presence of DHCA (100, 200 and 400 mg/kg) or dexamethasone (10mg/kg) daily, and sacrificed at day 5. Total RNAs were isolated from back skin and analyzed by quantitative RT-PCR using specific primers for IL-17A, IL-17F, IL-22, TNF-α, IL-6, IL-1β, S100A8, S100A9 and LL-37. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with Vehicle group.

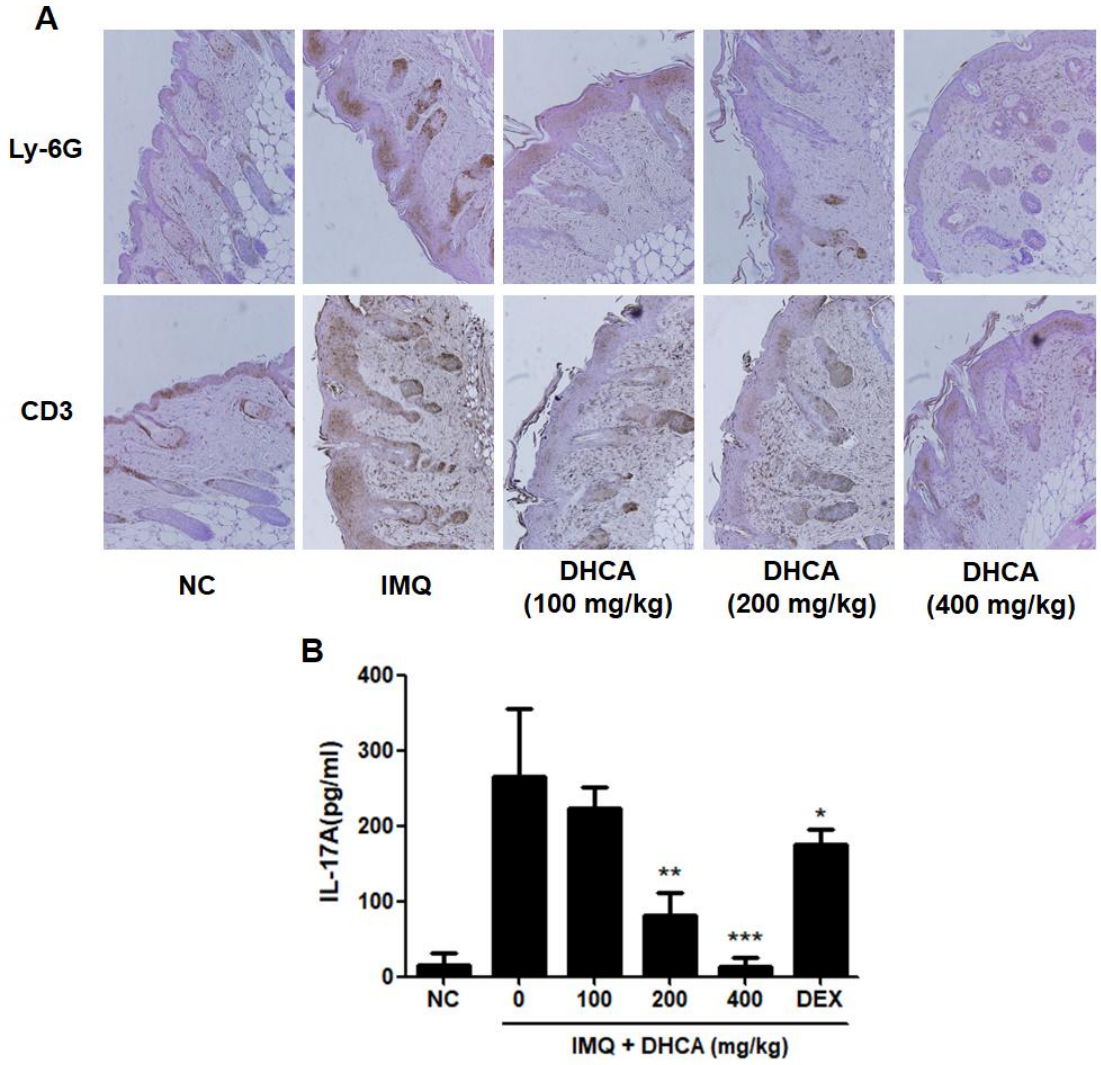


Figure 8. Effects of DHCA on the infiltration of inflammatory cells in psoriatic lesions. Mice were treated with IMQ in the presence of DHCA (100, 200 and 400 mg/kg) or dexamethasone (10mg/kg) daily, and sacrificed at day 5. (A) Effects of DHCA on infiltration of inflammatory cells. Immunohistochemical staining of the mouse back skin was performed using Ly-6G and CD3 antibodies as described in Materials and Methods section. (B) Effects of DHCA on the population of IL-17 producing cells. Primary skin cells were isolated using enzymatic digestion method. The skin cells were subsequently re-stimulated with PMA (20ng/mL) and Ionomycin (500ng/mL). Three days later, the culture supernatants were collected and subjected to IL-17 specific ELISA. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with Vehicle group.

PMA (20ng/mL) and Ionomycin (500ng/mL). Three days later, supernatants were collected and subjected to an IL-17 specific ELISA. As shown in Figure 8B, the protein level of IL-17 was highly increased in the IMQ-treated group, but treatment with DHCA lowered its level in a dose-dependent manner. Taken together, these data suggested that DHCA might inhibit the infiltration of IL-17-producing lymphocytes into the psoriatic lesions.

2.7 DHCA inhibits the production of chemokines in psoriatic lesions at early time-points.

Keratinocytes produce various chemokines such as CXCL1, CXCL8 and CCL20 that act as a chemoattractant to guide the migration of neutrophils and lymphocytes into the skin in response to external stimuli(Frink et al. 2007). To test the effects of DHCA on the production of such chemokines, the production kinetics of CXCL1, CXCL8 and CCL20 was investigated in mice that were treated daily with IMQ and DHCA. At appropriate times, serum and whole back skin lysates were analyzed by ELISA. In both blood and back skin, the blood level of CXCL1, CXCL8 and CCL20 was highly increased by IMQ, but such elevated levels were lowered by DHCA treatment in a dose-dependent manner (Figure 9A and B). Taken together, these data indicated that DHCA might inhibit the production of those chemokines that make inflammatory cells migrate to the psoriatic lesions.

3. Discussion

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from water-soluble extracts of *Cucurbita moschata*(Lee et al. 2012). It has previously been shown to contain anti-adipogenic(Lee et al. 2012), anti-inflammatory(Lee, Choi, and Kim 2015) and anti-oxidative stress activities(Lee and Kim 2014). More recently, DHCA has been shown to inhibit Th17 cell differentiation by regulating NF-κB activity(Lee et al. 2015). In this study, it was investigated

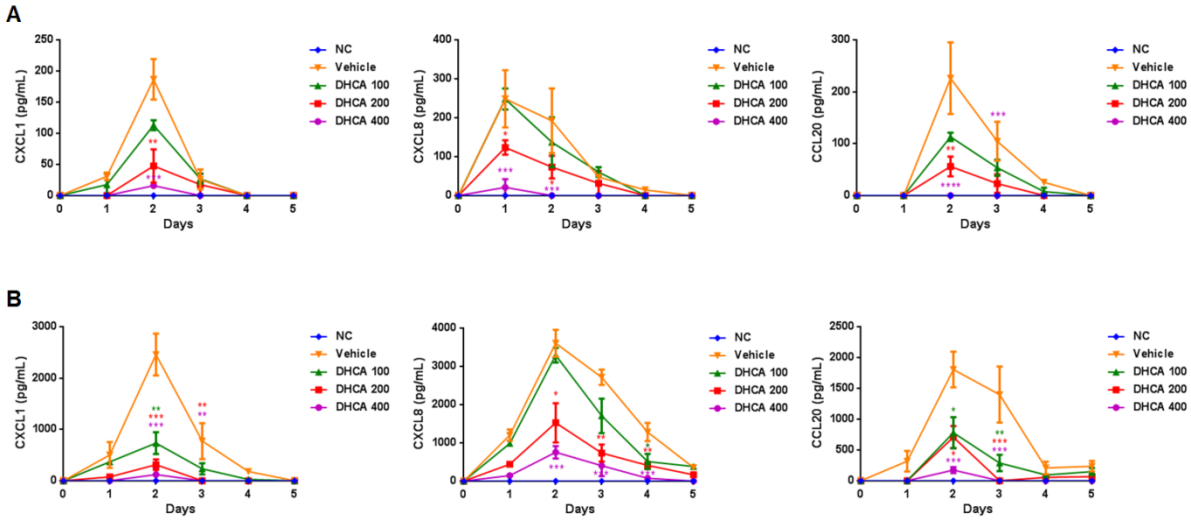


Figure 9. Effects of DHCA on the production of chemokines in serum and skin. 7-wk male Balb/c mice were topically administered followed by applying DHCA (100, 200 and 400 mg/kg) or dexamethasone (10mg/kg) in the presence of 62.5 mg of IMQ, on a daily basis, to the back skin of mice for 5 consecutive days. At appropriate times, mice were sacrificed, and serum and whole back skin lysates were isolated followed by ELISA. (A) Effects of DHCA on serum levels of CXCL1, CXCL8 and CCL20; (B) Effects of DHCA in whole back skin lysates. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ compared with Vehicle group.

whether synthetic DHCA could also reduce the inflammatory responses mediated by IL-17, using human keratinocyte cell line HaCaT. Our data showed that DHCA inhibited the IL-17-mediated cell proliferation and production of pro-inflammatory cytokines. Moreover, this lignan molecule suppressed the IL-17-mediated activation of p38 MAPK signaling pathway, resulting in the downregulation of expression of various related genes, such as for pro-inflammatory cytokines, chemokines and antimicrobial peptides. Consistent with these in vitro data, DHCA ameliorated the severity of the IMQ-induced psoriasis-like skin inflammation probably by reducing the production of pro-inflammatory cytokines and chemokines, thereby decreasing the infiltration of inflammatory cells in psoriatic lesions.

It has previously been reported that estrogen efficiently controls inflammatory responses induced by pro-inflammatory cytokines via the regulation of various transcription factors such as NF-κB and MAPKs(Ghisletti et al. 2005; Tashiro, Okamoto, and Bereiter 2009). Furthermore, estrogen has also been shown to inhibit the differentiation of Th17 cells and the production of IL-17, a potent inducer of inflammation through the down-regulation of RORγt transcription(Javadian et al. 2014). Although estrogen is widely considered to produce anti-inflammatory activities, according to our knowledge, the relationship between estrogen and IL-17-mediated signaling pathways has not been characterized to date. We recently found that DHCA interacted with both ERα and ERβ as an agonist for these receptors (submitted for publication). Taken together, these data suggest that estrogen may regulate IL-17-mediated actions through a mechanism similar to that of DHCA.

MAPK signaling is well known to play important roles in the regulation of various biological processes including mitosis, apoptosis and differentiation(Krens, Spaink, and Snaar-Jagalska 2006; Sun et al. 2015). It has been reported that IL-17-mediated activation of MAPKs plays an important role in the progression of inflammatory responses(Iyoda et al. 2010). Our data suggested that DHCA specifically suppressed IL-17-induced p38 MAPK pathway, but not the other two pathways involving ERK1/2 and JNK, indicating that DHCA might interact with upstream molecules of the p38 pathway, for example MAPK kinases (MAPKKs) like MEK3 and MEK6.

It was recently reported that IL-22, a member of the IL-10 cytokine family, might contribute to the development of psoriasis(Fujita 2013). For example, elevated levels of IL-22 were observed in plasma and psoriatic skin lesions from patients with psoriasis, and its level correlated positively with severity of the disease(Hao 2014). In addition, IL-22-deficient mice were shown to be protected from psoriasis-like skin inflammation induced by IMQ(Van Belle et al. 2012). We showed that the RNA level of IL-22 was increased by almost 320-fold in IMQ-treated mice, but treatment with 100mg/kg DHCA reduced this effect by 11-fold. Therefore, IL-22 may also play a role(s) in the ameliorating effects of DHCA on IMQ-induced psoriasis-like skin inflammation. Further experiments would be necessary to determine the effects of DHCA on IL-22-mediated actions.

DHCA treatment was shown to reduce the number of IL-17 secreting cells in psoriatic lesions, but the exact cell type(s) is not yet clear. Various cell types, including Th17, γδ T cell and neutrophil, were reported to produce IL-17(Cua and Tato 2010; Coffelt et al. 2015). Identifying the cell types affected by DHCA will be important in understanding the action mechanism(s) of DHCA.

Our data indicated that DHCA reduced IL-17-induced inflammatory responses by affecting the p38 MAPK pathway. It was recently reported that DHCA could inhibit the differentiation of IL-17 producing Th17 cells from naïve CD4+ T cells, and also that it suppressed the production of pro-inflammatory molecules in Raw264.7, a macrophage cell line. In summary, DHCA seems to contain potent anti-inflammatory activities by controlling the production and action of pro-inflammatory molecules. Taken together, our results indicate that DHCA may be developed as an efficient therapeutic for psoriasis by controlling various inflammatory responses through its multiple actions.

Chapter IV

Anti-osteoclastogenic Effects of DHCA in RAW264.7 Cells

1. Background

Osteoporosis is a skeletal disease characterized by a reduced density and quality of bones leading to increased susceptibility of fractures(Kanis 2002; Gardner et al. 2006). Osteoporosis has become a serious health problem due to its prevalence(Wright et al. 2014). Currently, more than 200 million people are estimated to be suffering from this disease worldwide(Wilkins 2007), with the number of patients expected to grow rapidly with the rise in the elderly population(Vondracek and Linnebur 2009). There are several FDA-approved drugs available for osteoporosis such as denosumab(Iqbal, Sun, and Zaidi 2010), ibandronate(Bauss and Schimmer 2006), and raloxifene(Messalli and Scaffa 2010). However, because of limitations of their efficacy and safety, there is still a huge unmet medical need for a solution to this degenerative disease.

Excessive bone resorption is a major step in the pathogenesis of osteoporosis(Riggs 2000), and osteoclasts are responsible for this process by degrading the bone matrix(Everts et al. 2006). Several signaling molecules involved in osteoclastogenesis, meaning the differentiation process of osteoclasts from precursor cells, have been implicated as a promising target for the prevention or treatment of osteoporosis. Interaction of RANKL with its receptor, RANK, initiates the osteoclast differentiation by activating various signaling pathways, such as those involving NF-κB(Soysa and Alles 2009) and MAPKs(Lee et al. 2016). NFATc1, a downstream transcription factor in NF-κB(Kim and Kim 2014) and MAPKs(Choi et al. 2014; Kim and Kim 2014) pathways, plays a master role in the regulation of various genes involved in osteoclast functions(Takayanagi et al. 2002), such as MMP-9 (matrix metalloproteinase-9), DC-STAMP (dendrocyte expressed seven transmembrane protein) and cathepsin K. In experiments involving AMPK-knockout mice, the AMPK signaling pathway has also been shown to play a key role in osteoclastogenesis by promoting bone formation or reducing bone mass(Shah et al. 2010).

It has been well established that estrogen deficiency during menopause generates significant bone loss(Riggs 2000). Estrogen can suppress bone resorption by directly inhibiting

RANKL-induced osteoclastogenesis through the control of c-Jun activity(Shevde et al. 2000). A bulk of studies also revealed that estrogen could regulate osteoclastogenesis by down-regulating the production of TNF-α, IL-1β and IL-6, resulting in the enhanced production of RANKL and M-CSF (macrophage colony-stimulating factor) in stromal cells(Clowes, Riggs, and Khosla 2005). Such effects of estrogen on the prevention of bone loss are strongly supported by data from experiments involving knockout mice lacking ERα(Nakamura et al. 2007). Consequently, a variety of phytoestrogens and SERMs have been tested for their possible use in treating postmenopausal osteoporosis(Al-Anazi et al. 2011).

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from water-soluble extracts of *Cucurbita moschata*(Lee et al. 2012). DHCA had been thought to be a member of the phytoestrogen family. Indeed, our group previously reported that synthetic DHCA contains a wide range of estrogen-like activities with effects such as anti-adipogenic(Lee et al. 2012), anti-inflammatory(Lee, Choi, and Kim 2015) and anti-oxidative effects(Lee and Kim 2014) by regulating C/EBPβ, NF-κB and AMPK signaling pathways, respectively. In this study, we hypothesized that DHCA might have a beneficial effect(s) on preventing osteoclastic bone loss by acting as an estrogen receptor agonist. The effects of DHCA on RANKL-induced osteoclastogenesis *in vitro* have been investigated, and the underlying mechanisms also have been studied.

2. Results

2.1 DHCA inhibits RANKL-induced osteoclast differentiation of RAW264.7 cells with no cytotoxic effect.

RAW264.7 cells are the murine pre-osteoclast cell line that can differentiate into osteoclasts when stimulated with RANKL(Shevde et al. 2000). TRAP (tartrate-resistant acid

phosphatase) is a specific marker for mature osteoclasts(Ballanti et al. 1997). To induce osteoclastogenesis, RAW264.7 cells were treated with RANKL (50ng/mL) and various concentrations of DHCA (10, 20 and 40 μM) for five days. TRAP-positive multi-nucleated cells were counted, and TRAP activity was measured. As shown in Figure 10A–C, DHCA treatment reduced the number and size of TRAP-positive multi-nucleated cells in a dose-dependent manner. Consistently, TRAP activity was also decreased by DHCA in a similar way (Figure 10D).

To be certain, effects of DHCA on cell viability were measured. RAW264.7 cells were cultured with or without RANKL in the presence of DHCA, and cell viability was examined by MTT assay. As shown in Figure 10E, DHCA had little effect on cell viability in all concentrations. DHCA did not have any cytotoxic effects during 72 hours regardless of the presence of RANKL.

2.2 DHCA suppresses bone resorption activity by inhibiting MMP activity and production.

Mature osteoclasts produce proteinases such as MMP-2 and MMP-9, which play an important role in bone resorption(Ohshiba et al. 2003). To investigate the effects of DHCA on bone resorption activity, pit formation assay was performed. RAW264.7 cells were seeded on an osteoassay plate and co-treated with RANKL and various concentrations of DHCA for 7 days. As shown in Figure 11A, DHCA treatment inhibited RANKL-induced bone resorption in a dose-dependent manner.

To test the effects of DHCA on the activity of MMPs induced by RANKL, RAW264.7 cells were co-cultured with RANKL and various concentrations of DHCA for 24 hours, and culture supernatants were subjected to gelatin zymography. DHCA treatment reduced RANKL-induced MMP-2 and MMP-9 activities in a dose-dependent manner (Figure 11B). Next, we further tested whether DHCA can also suppress the RANKL-induced production of MMPs. RAW264.7 cells were co-cultured with RANKL and various concentrations of DHCA for

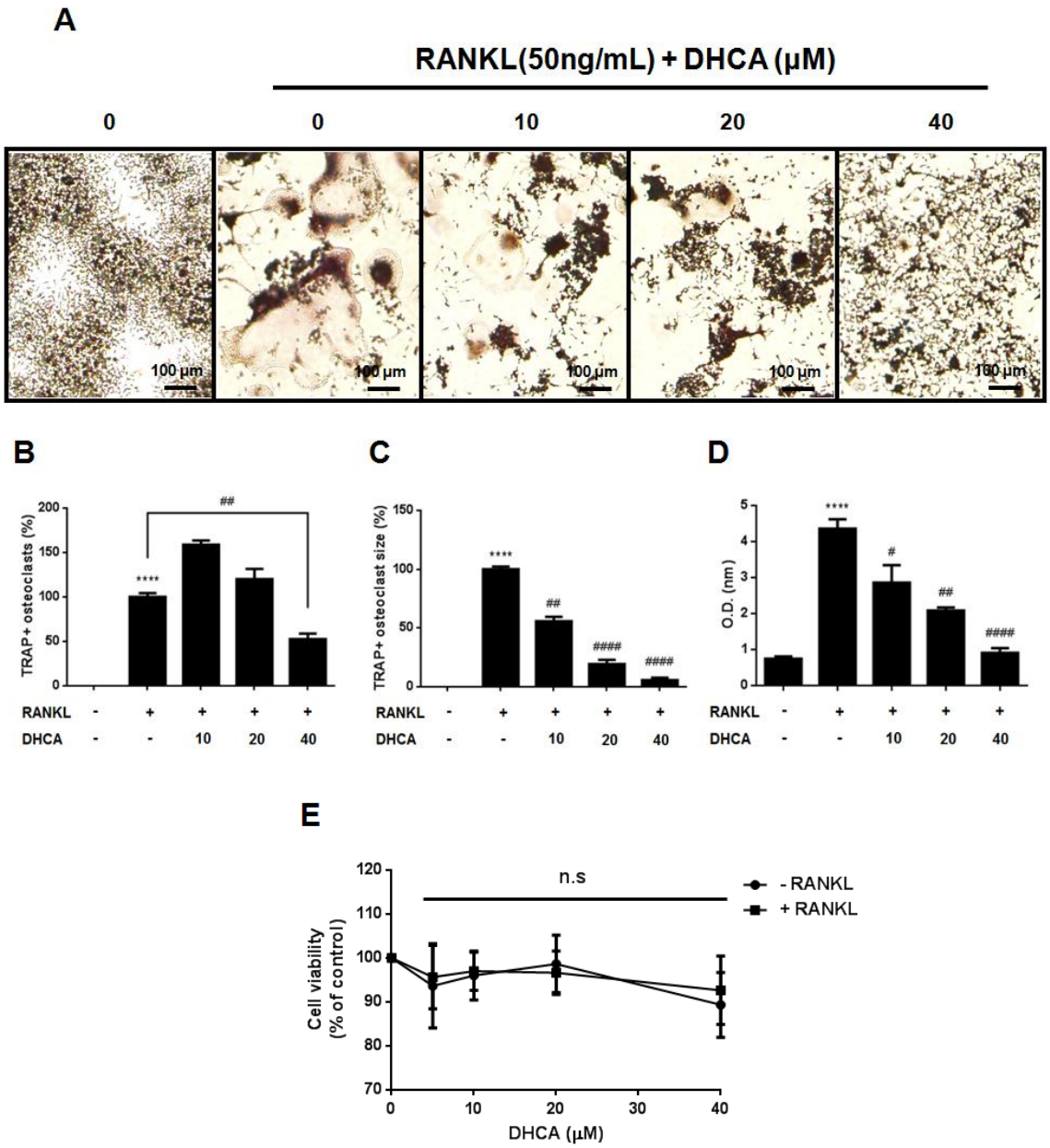


Figure 10. Effects of DHCA on RANKL-induced osteoclast differentiation. (A) TRAP-positive multi-nucleated cells were visualized by TRAP stain; (B) TRAP-positive multi-nucleated cells were counted under the microscope; (C) Size of TRAP-positive multi-nucleated cells was measured; (D) TRAP activity was measured at 450nm following TRAP activity assay. RAW264.7 cells were treated with RANKL (50ng/mL), and cultured in the presence of DHCA for 5 days; (E) RAW264.7 cells were treated with or without RANKL and various concentrations of DHCA for 72 hours. Cells were then subjected to MTT assay as described in the Method section. Values represent the mean ± S.E.M. of three independent experiments. $^{****}p<0.0001$ compared with control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$ compared with that treated with RANKL alone.

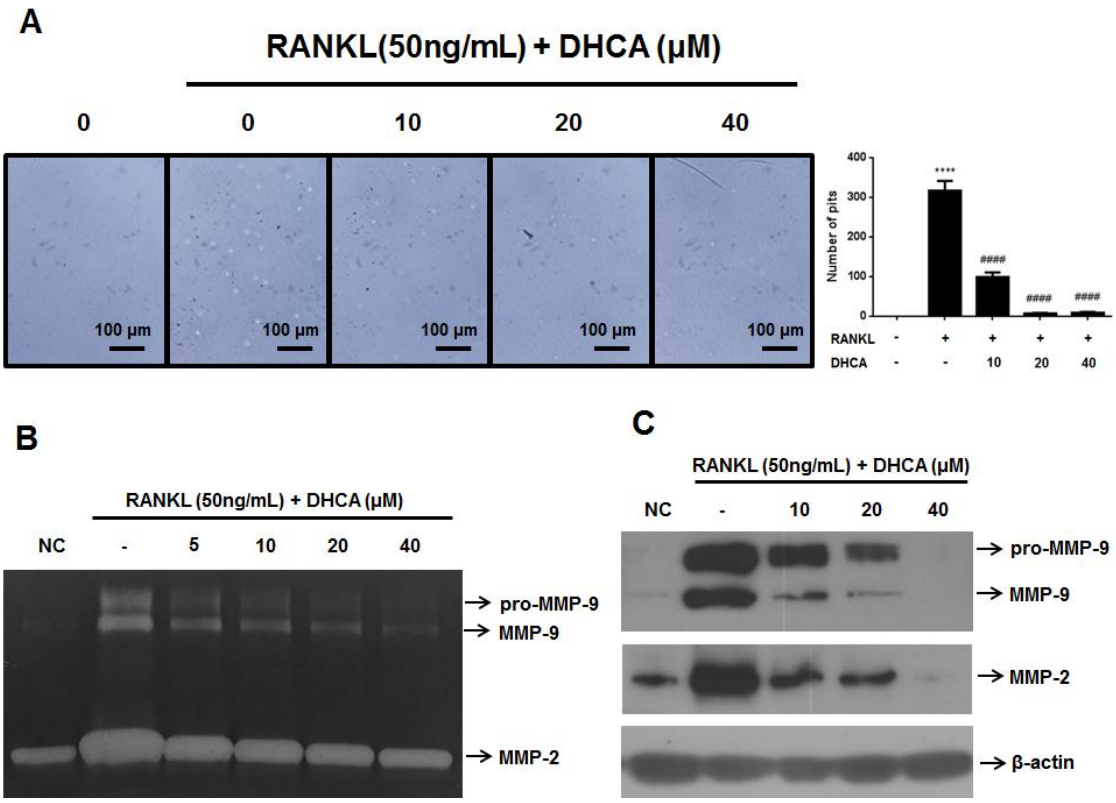


Figure 11. Effects of DHCA on bone resorptive activity of osteoclast. (A) Pit formation on the osteoassay plate was observed by optical microscopy. RAW264.7 cells were treated with RANKL (50ng/mL), and cultured in the presence of DHCA for 5 days. After that, pit formation was measured as described in the Materials and Method section; (B) Activities of MMP-2 and MMP-9 were measured by gelatin zymography; (C) Protein levels of MMP-2 and MMP-9 were measured by western blot. RAW264.7 cells were treated with RANKL (50ng/mL), and cultured in the presence of DHCA for 24 hours. Values represent the mean ± S.E.M. of three independent experiments. $^{****}p<0.0001$ compared with control; $^{####}p<0.0001$ compared with that treated with RANKL alone.

24 hours, and total cell lysates were subjected to western blot analysis. RANKL treatment increased the amount of the pro- and the active- form of MMP-9, but DHCA reduced it in a dose-dependent manner (Figure 11C). Similarly, RANKL-induced production of MMP-2 was also decreased by DHCA treatment (Figure 11C). Taken together, these data indicated that DHCA might reduce bone resorption activity of mature osteoclasts by inhibiting the activity and production of MMP-2 and MMP-9.

2.3 DHCA inhibits the expression of osteoclastogenic genes.

NFATc1 is a well-known transcription factor playing a major role in osteoclastogenesis. RANKL stimulation to pre-osteoclasts increases expression of NFATc1, leading to the activation of various osteoclastogenic genes involved in differentiation and functions of osteoclasts such as TRAP, c-Fos, DC-STAMP, MMP-9, and Cathepsin K(Liou et al. 2013). To test the effects of DHCA on these genes, RAW264.7 cells were co-cultured with RANKL and DHCA for 24 hours, and the RNA level was measured by quantitative RT-PCR. DHCA treatment decreased the RNA level of NFATc1 and c-Fos, along with other osteoclast-specific genes such as TRAP, MMP-9, DC-STAMP and Cathepsin K, in a dose-dependent manner (Figure 12).

2.4 DHCA suppresses the RANKL-induced production of inflammatory cytokines.

Inflammatory cytokines such as TNF-α, IL-6, and IL-1β play important roles in osteoclast differentiation and functions. To test the effects of DHCA on inflammatory cytokine production, RAW264.7 cells were cultured with RANKL and various concentrations of DHCA for 48 hours, and the levels of TNF-α, IL-6, and IL-1β in the culture supernatants were measured by ELISA. DHCA treatment inhibited the RANKL-induced production of TNF-α, IL-6, and IL-1β in a dose-dependent manner (Figure 13A). We also measured the effects of DHCA on the RNA level of these molecules by quantitative RT-PCR. As shown in Figure 13B,

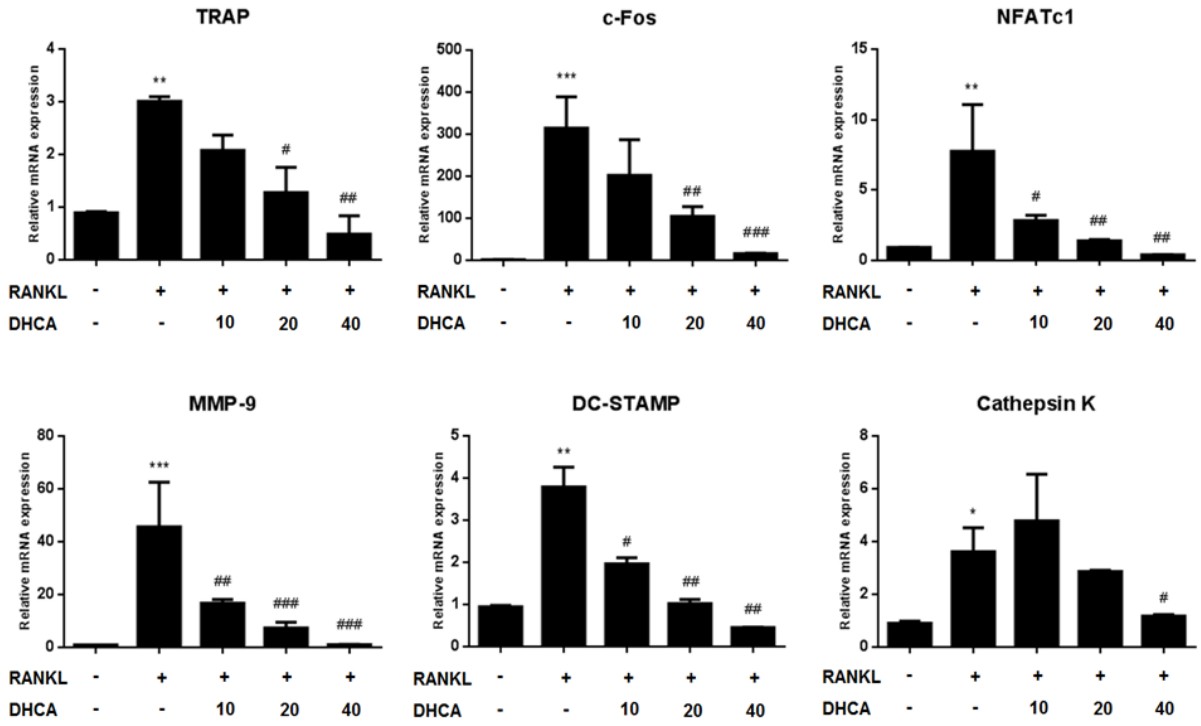


Figure 12. Effects of DHCA on osteoclastogenic gene expression during RANKL-induced osteoclastogenesis. Total RNAs were prepared followed by quantitative RT-PCR using specific primers for TRAP, c-Fos, NFATc1, MMP-9, DC-STAMP, and Cathepsin K. RAW264.7 cells were treated with RANKL (50ng/mL), and cultured in the presence of DHCA for 24 hours. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with that treated with RANKL alone.

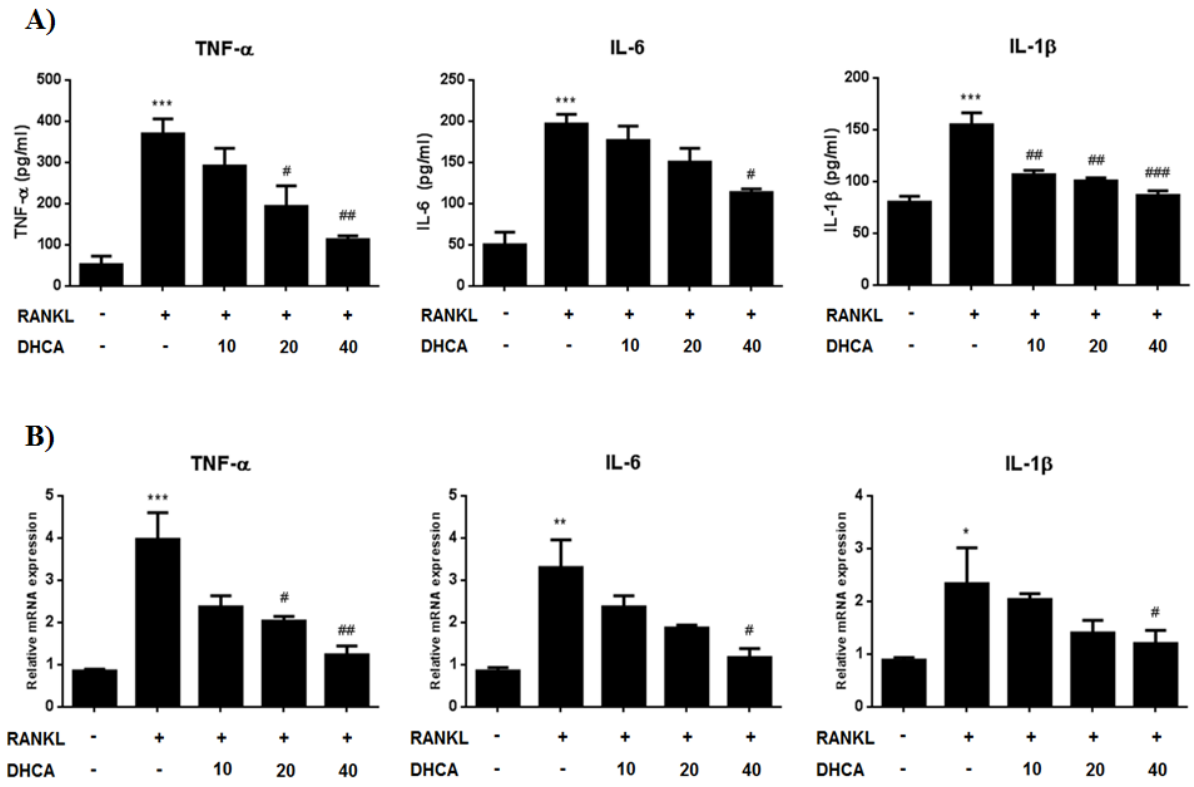


Figure 13. Effects of DHCA on RANKL-induced inflammatory cytokine production and expression. (A) Effects of DHCA on TNF-α, IL-6 and IL-1β production; (B) Effects of DHCA on TNF-α, IL-6 and IL-1β expression. RAW264.7 cells were treated with RANKL (50ng/mL), and cultured in the presence of DHCA for 48 hours. Values represent the mean ± S.E.M. of triplicate samples. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with that treated with RANKL alone.

RANKL-induced expression of TNF-α, IL-6, and IL-1β were markedly reduced by DHCA treatment in a dose-dependent manner. These data suggested that DHCA could effectively inhibit the RANKL-induced production of inflammatory cytokines at the RNA level.

2.5 DHCA suppresses RANKL-induced NFATc1 and c-Fos production via NF-κB and p38 MAPK inhibition.

During RANKL-induced osteoclastogenesis, NFATc1 and c-Fos have been known to be activated by NF-κB and MAPK signaling pathways, which are critical steps in the differentiation of osteoclasts(Huang et al. 2006; Boyce et al. 2005). To test the effects of DHCA on the RANKL-induced protein expression of transcription factors, RAW264.7 cells were co-treated with RANKL and DHCA for 24 hours, and the levels of NFATc1 and c-Fos were measured by Western blot. As shown in Figure 14A, DHCA treatment inhibited RANKL-induced production of NFATc1 and c-Fos in a dose-dependent manner.

To further investigate the effects of DHCA on the RANKL-induced signaling pathway, RAW264.7 cells were co-treated with RANKL and DHCA for 30 minutes, and the phosphorylation status of Akt-IKK and MAPKs was determined by Western blot. When cells were treated with RANKL, Akt was efficiently phosphorylated, but its level was highly decreased by DHCA treatment (Figure 14B). It was also found that phosphorylation of IKK, a downstream molecule of Akt, was suppressed in the presence of DHCA (Figure 14B). Consistent with these results, RANKL-induced degradation of the IκB proteins was recovered by DHCA (Figure 14B). These data indicated that DHCA might effectively control the Akt-IKK-NF-κB axis activated by RANKL.

The phosphorylation status of MAPKs was also affected by DHCA. RANKL stimulation increased phosphorylation of JNK, p38, and ERK (Figure 14C), and DHCA treatment affected p38 phosphorylation, but did not affect JNK and ERK (Figure 14C). Taken together, DHCA negatively controlled the RANKL signaling pathway by inhibiting the

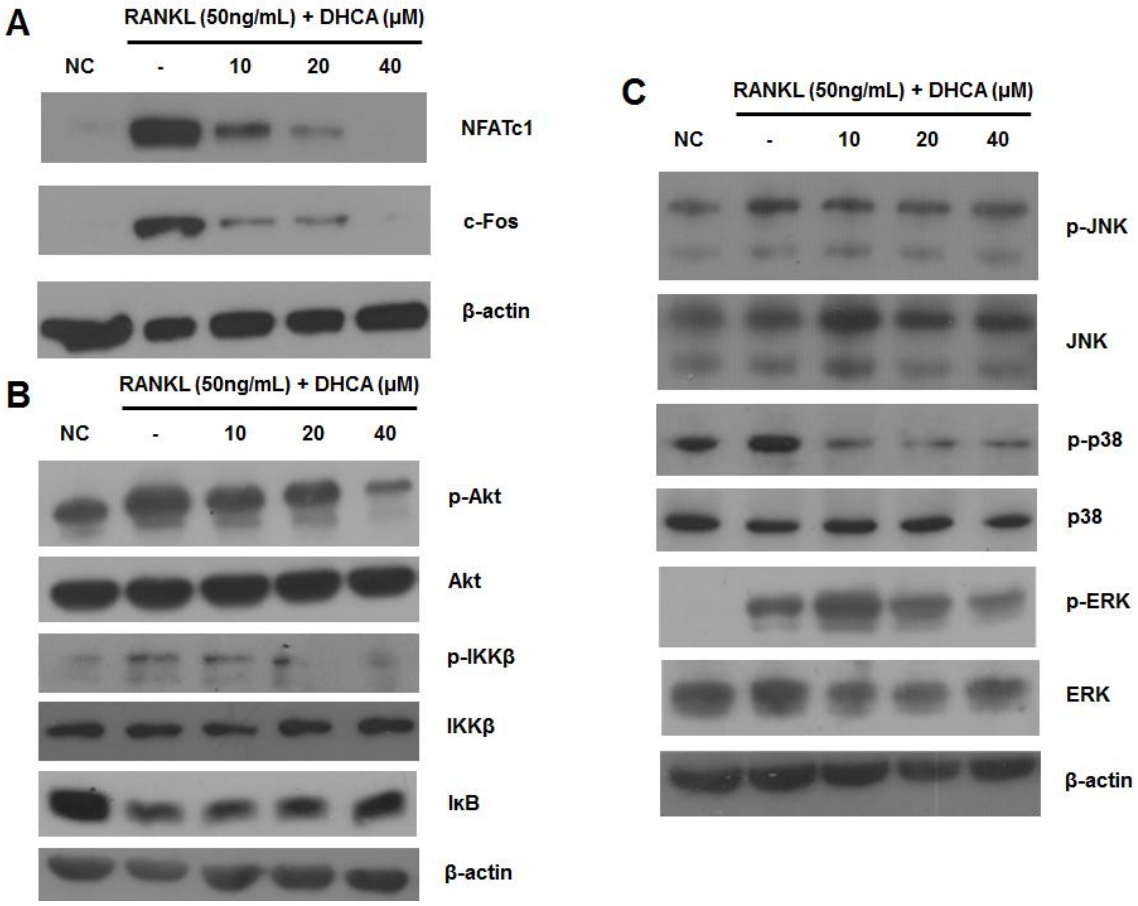


Figure 14. Effects of DHCA on NF-κB and MAPK signaling pathways. (A) Proteins involved in the NF-κB signaling pathway; (B) Proteins involved in the MAPKs signaling pathway. RAW264.7 cells were treated with RANKL (50ng/mL), and cultured in the presence of DHCA for 30 min. Total protein were prepared following by western blot using antibodies specific for respective proteins.

Akt-IKK-NF-κB axis and the p38 MAPK.

2.6 DHCA attenuated RANKL-induced osteoclastogenesis by activating AMPK.

It has been previously reported that AMPK acts as a negative regulator of RANKL-induced osteoclastogenesis, via inactivation of various downstream signaling elements such as p38, JNK, NF-κB, Akt, CREB, c-Fos, and NFATc1(Shah et al. 2010; Lee et al. 2010). Therefore, the effect of DHCA was investigated on AMPK. When RAW264.7 cells were treated with RANKL alone, the level of phosphorylated AMPK was increased, and this effect was further enhanced upon DHCA treatment (Figure 15A, compare lanes 2 and 5).

To confirm the relationship between AMPK and DHCA, RAW264.7 cells were transfected with siRNA against AMPKα1 followed by treatment with RANKL and DHCA. As shown in Figure 15C–F, the anti-osteoclastogenic effects of DHCA were suppressed when cells were transfected with siRNA. Similarly, the DHCA-mediated reduction of TRAP, NFATc1, and c-Fos expressions were also inhibited by siRNA (Figure 15G). These data indicated that DHCA might suppress osteoclastogenesis by activating AMPK.

2.7 Effects of DHCA on activating AMPK were mediated by the estrogen signaling pathway.

A significant number of phytochemicals are known to interact with estrogen receptors and act as SERMs(Oseni et al. 2008). Furthermore, estradiol demonstrates anti-osteoclastogenic activity via activation of AMPK(Shevde et al. 2000). To investigate the possible mechanisms by which DHCA activates AMPK, inhibitor assay targeting the upstream factors of AMPK was performed. RAW264.7 cells were co-treated with RANKL, DHCA, and various concentrations of AMPK-upstream inhibitors for 5 days, and TRAP activity was measured. As shown in Figure 16A–C, PKA inhibitor H89 (1-10 μM) and CaMKKβ inhibitor STO609 (1-10 μM) did not

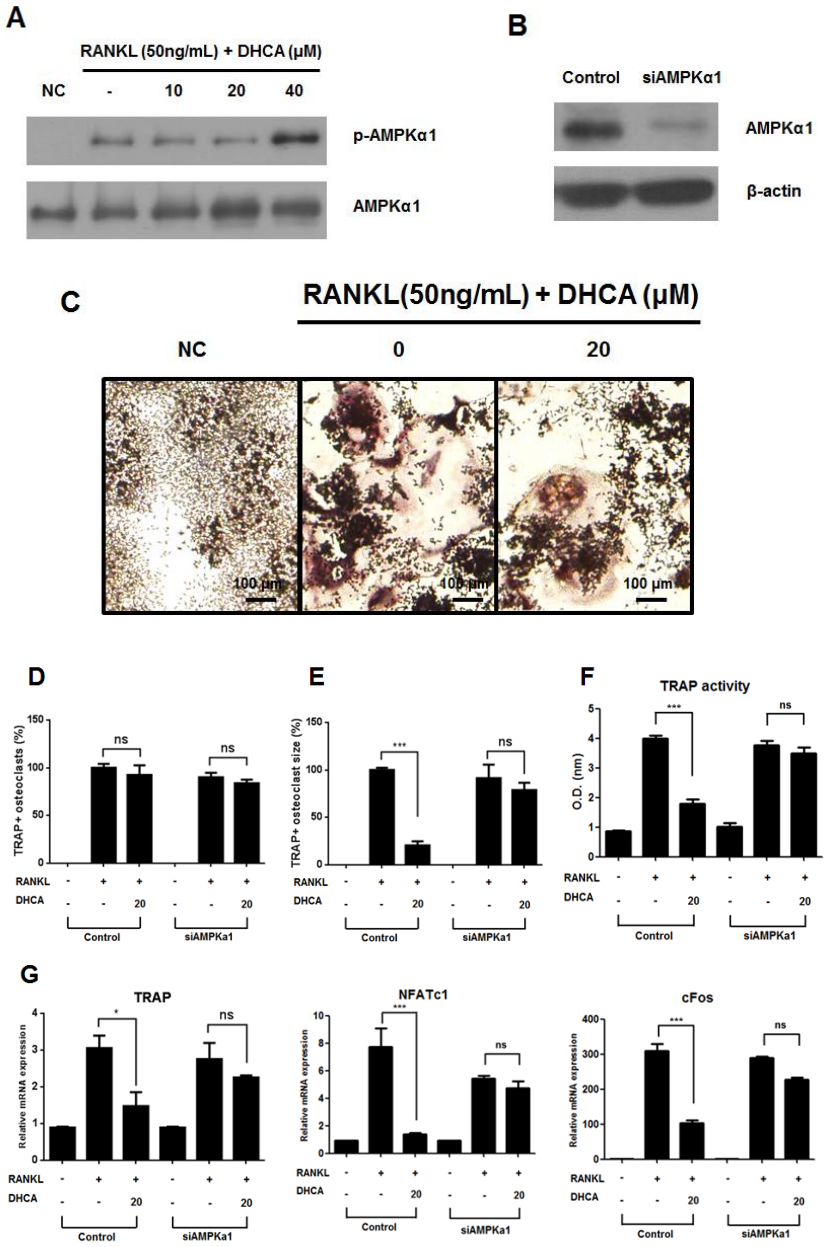


Figure 15. Effects of DHCA on AMPK signaling pathway during RANKL-induced osteoclast differentiation. (A) Phosphorylation status of AMPKα1 was enhanced by DHCA during RANKL-induced osteoclast differentiation; (B) AMPKa1 protein was knocked down by siRNA; (C) TRAP-positive multi-nucleated cells were visualized by TRAP stain; (D) TRAP-positive multi-nucleated cells were counted under the microscope; (E) Size of TRAP-positive multi-nucleated cells was measured; (F) TRAP activity was measured at 450nm following TRAP activity assay after 5 days; (G) Osteoclastogenic gene expression were measured by quantitative RT-PCR after 24 hours. RAW264.7 cells were transfected with AMPKα1 siRNA or control siRNA, then were co-treated with RANKL (50ng/mL) and DHCA (20μM). Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{***}p<0.001$ compared with that treated with RANKL alone.

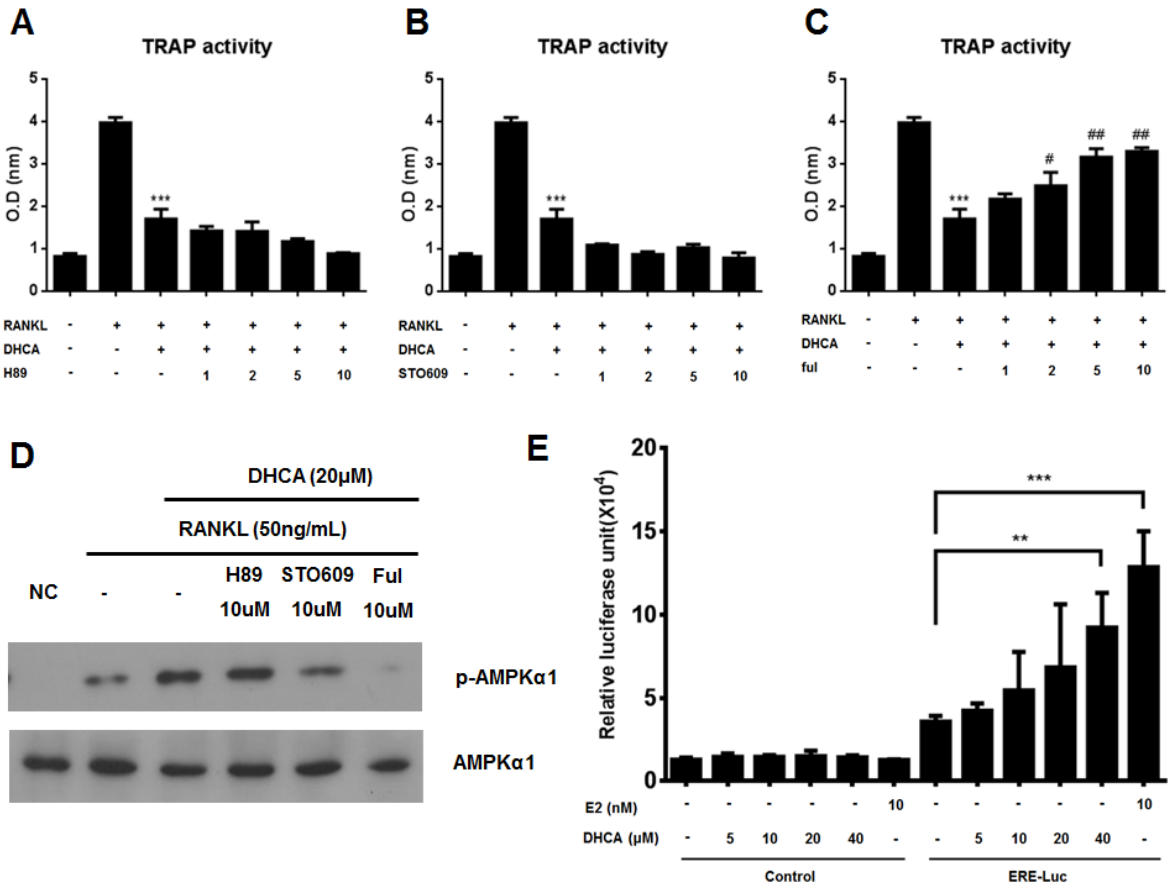


Figure 16. Effects of DHCA on estrogen receptor signaling pathway. (A-C) TRAP activity was measured at 450nm following TRAP activity assay after 5 days; (D) Phosphorylation status of AMPKα1 was measured. RAW264.7 cells were co-cultured with RANKL, DHCA (20μM), and various concentrations of H89, STO609, and fulvestrant; (E) Luciferase activity was measured. RAW264.7 cells were transfected with control or luciferase reporter plasmid containing sequences for ERE, then were treated with various concentrations of DHCA or E2 (10nM) for 6 hours. Total protein were prepared and the activity of luciferase was measured using luminometer. Values represent the mean ± S.E.M. of three independent experiments. $^{***}p<0.001$ compared with that treated with RANKL alone; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with that treated with RANKL and DHCA.

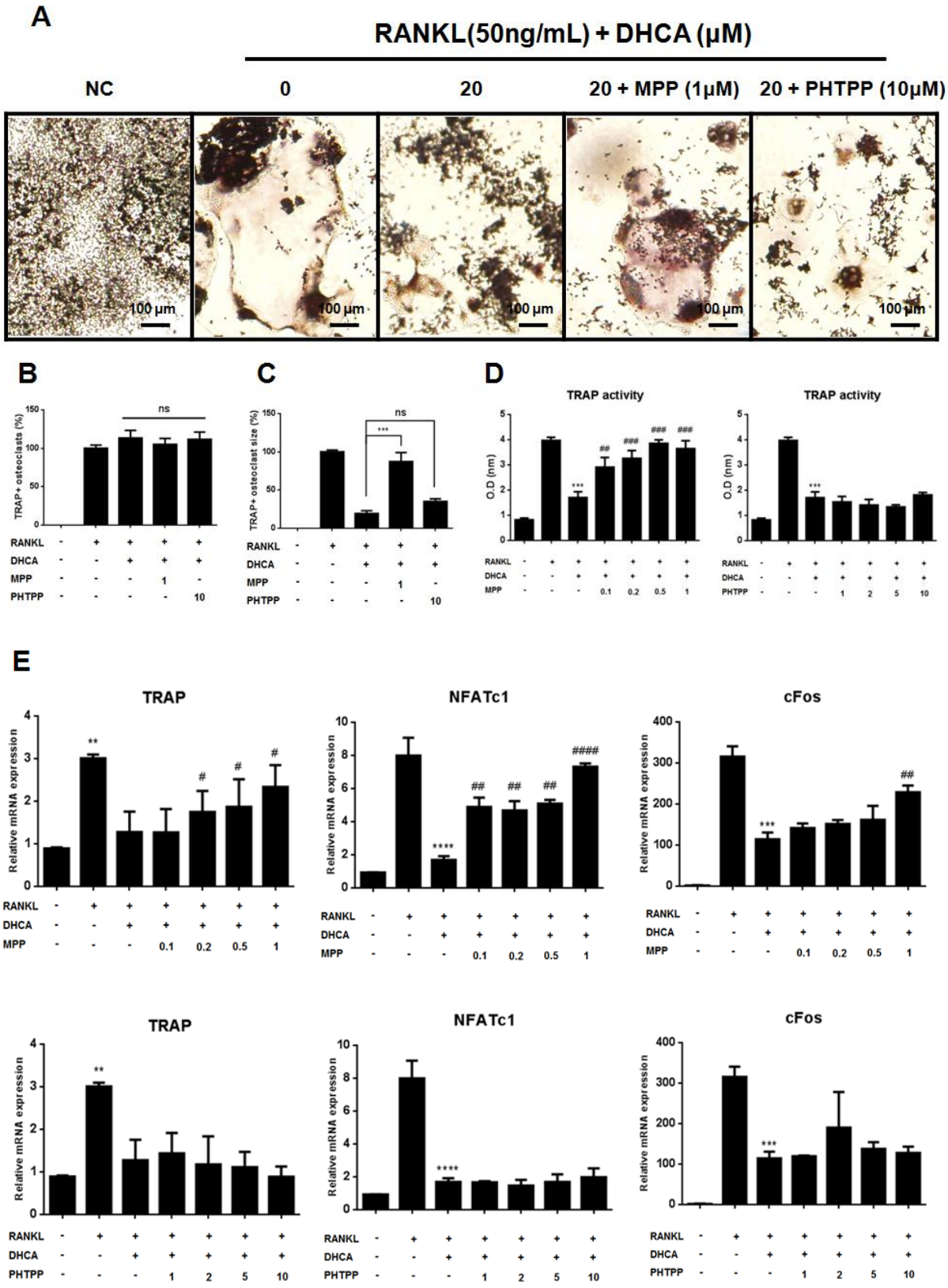
interfere with DHCA activity, while estrogen receptor antagonist fulvestrant (1-10 μM) diminished the effects of DHCA. Consistently, the level of phosphorylated AMPK was decreased when fulvestrant was co-treated with DHCA, but H89 and STO609 did not alter the level of phosphorylated AMPK (Figure 16D). Therefore, the effects of DHCA on AMPK activation appeared to be mediated by the estrogen receptor, not PKA or CaMKKβ.

To test the effects of DHCA on the estrogen-induced signaling pathway, RAW264.7 cells were transfected with a luciferase reporter plasmid containing nucleotide sequences for estrogen responsive element (ERE). Twenty-four hours later, transfected cells were treated with estradiol or DHCA for 6 hours. Total proteins were extracted, and relative luciferase unit was measured by luminometer. When cells were treated with DHCA, the level of luciferase activity was increased by DHCA in a dose-dependent manner (Figure 16E).

2.8 Anti-osteoclastogenic effects of DHCA were mediated by ERα, but not ERβ.

There are two different forms of the estrogen receptor, usually referred to as ERα and ERβ, and they are known to regulate osteoclastogenesis in a different way(Bord et al. 2001; Nakamura et al. 2007). To investigate which of the two estrogen receptors DHCA interacts with and exerts its effects, antagonists that target the specific estrogen receptor were used. RAW264.7 cells were treated with various concentrations of ERα specific antagonist MPP (0.1-1 μM) or ERβ specific antagonist PHTPP (1-10 μM) in the presence of RANKL and DHCA, then RANKL-induced osteoclastogenesis and osteoclastogenic gene expression were measured. As shown in Figure 17A–E, MPP abolished DHCA-mediated inhibition of osteoclastogenesis and expression of osteoclastogenic transcription factor, while PHTPP had no effect.

To confirm the relationship between estrogen receptors and DHCA, RAW264.7 cells were transfected with siRNA against ERα or ERβ followed by treatment with RANKL and DHCA. Anti-osteoclastogenic effects of DHCA were suppressed when cells were transfected with ERα siRNA (Figure 17G–K), whereas these effects were not affected by transfection with ERβ siRNA (Figure 17M-Q). Taken together, these data indicated that DHCA might interact


A
RANKL(50ng/mL) + DHCA (μM)
NC
0
20
20 + MPP (1μM)
20 + PHTPP (10μM)
100 μm
B
TRAP+ osteoclasts (%)
ns
RANKL
DHCA
MPP
PHTPP
C
TRAP+ osteoclast size (%)
ns
***
D
TRAP activity
O.D (nm)
TRAP activity
O.D (nm)
E
TRAP
NFATc1
cFos
Relative mRNA expression
TRAP
NFATc1
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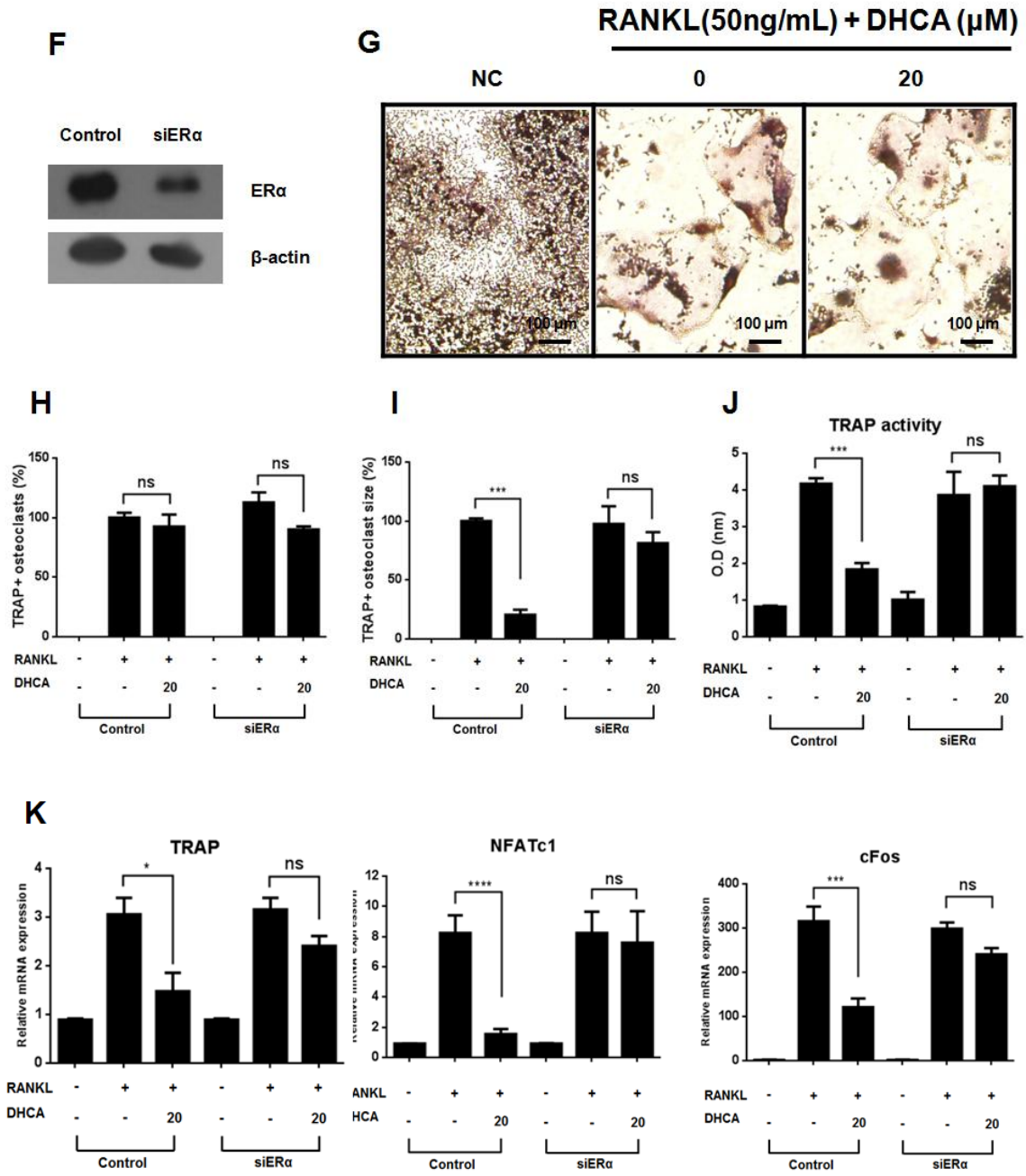
Relative mRNA expression


F
Control
siERα
ERα
β-actin
G
RANKL(50ng/mL) + DHCA (μM)
NC
0
20
100 μm
H
TRAP+ osteoclasts (%)
ns
RANKL
DHCA
Control
siERα
I
TRAP+ osteoclast size (%)
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J
TRAP activity
O.D (nm)
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Relative mRNA expression
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NFATc1
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cFos
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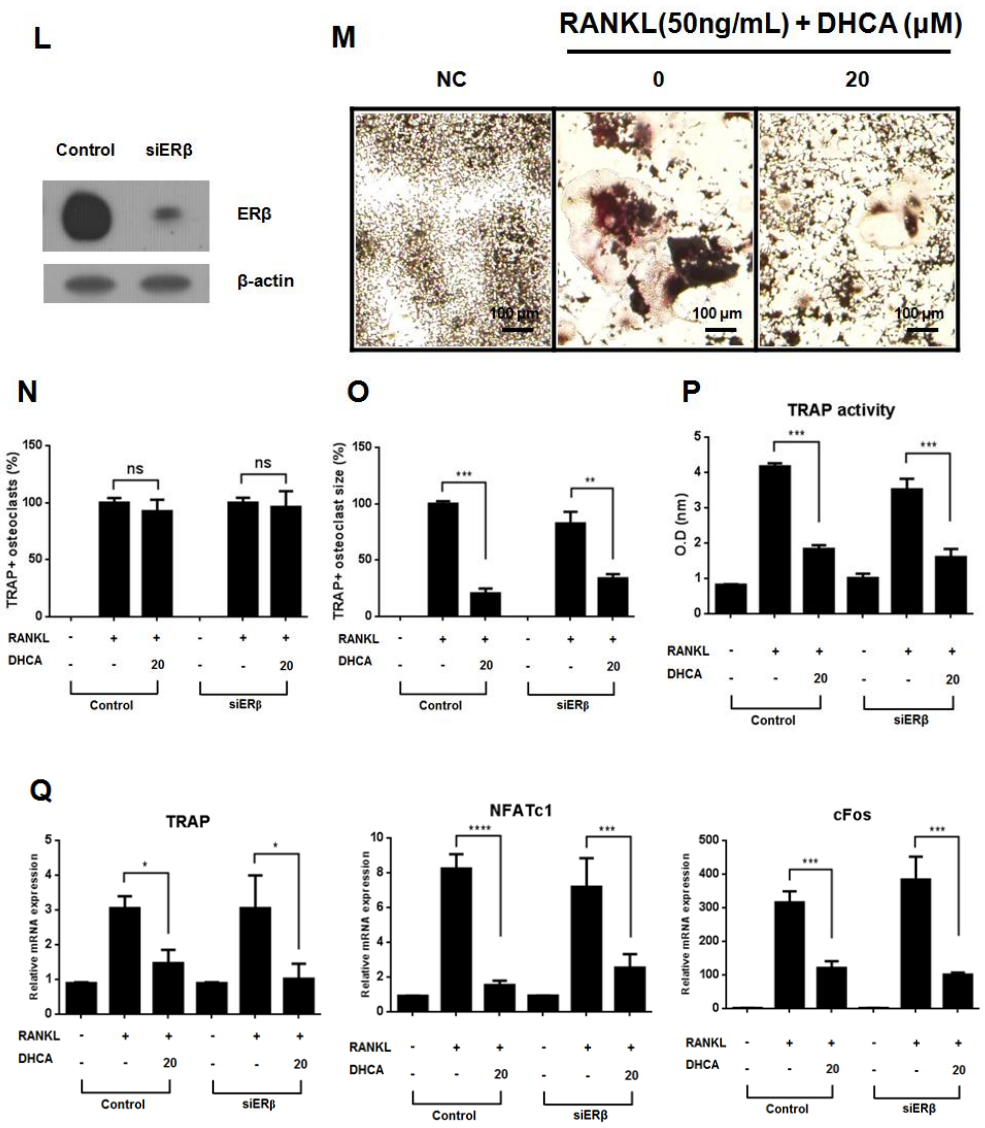

Figure 17. Anti-osteoclastogenic effects of DHCA were mediated by ERα, but not ERβ. (A) TRAP-positive multi-nucleated cells were visualized by TRAP stain; ; (B, G, M) TRAP-positive multi-nucleated cells were counted under the microscope; (C, H, N) Size of TRAP-positive multi-nucleated cells was measured; (D, I, O) TRAP activity was measured at 450nm following TRAP activity assay after 5 days; TRAP activity was measured at 450nm following TRAP activity assay after 5 days; (E, K, Q) Osteoclastogenic gene expression were measured by quantitive RT-PCR after 24 hours; (F) ERα protein was knocked down by siRNA; (L) ERβ protein was knocked down by siRNA; Raw264.7 cells were co-treated with RANKL (50ng/mL) and various concentrations of MPP (0.1-1μM) or PHTPP (1-10μM), and cultured in the presence of DHCA (20μM); Raw264.7 cells were transfected with ERα, ERβ siRNA or control siRNA, then were co-treated with RANKL (50ng/mL) and DHCA (20uM). Values represent the mean ± S.E.M. of triplicate samples. $^{***}p<0.001$ compared with that treated with RANKL alone; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with that treated with RANKL and DHCA.

with ERα, but not ERβ, to inhibit RANKL-induced osteoclast differentiation.

3. Discussions

DHCA is a lignan compound isolated from water-soluble extracts of *Cucurbita moschata*(Lee et al. 2012). It was previously shown to contain potent anti-adipogenic(Lee et al. 2012), anti-inflammatory(Lee, Choi, and Kim 2015) and anti-oxidative activities(Lee and Kim 2014) in fibroblast, macrophage and lymphocyte cell types. DHCA is a member of the phytoestrogens, while RANKL-induced osteoclastogenesis has been shown to be inhibited by estrogen. In this study, we investigated the effects of DHCA on RANKL-induced osteoclastogenesis in RAW264.7 cells. DHCA reduced the number of TRAP-positive multinucleated cells as well as inhibited the activity of TRAP. This lignan molecule decreased the expression of various genes involved in osteoclastogenesis such as NFATc1, c-Fos, TRAP, MMP-9, DC-STAMP and cathepsin K, and suppressed the signaling pathways involving p38 MAPK and NF-κB induced by RANKL. Furthermore, DHCA increased the level of phosphorylated AMPK, while the above anti-osteoclastogenic effects of DHCA were diminished when AMPKα1 expression was knocked down with siRNA. The AMPK-activating effect of DHCA was attenuated by inhibition of ERα, not ERβ.

MAPKs are protein kinases involved in the control of cellular responses to extracellular stimuli, such as growth factors, heat shock proteins and pro-inflammatory cytokines, and regulate a variety of biological processes including mitosis, apoptosis and differentiation(Pearson et al. 2001). The most studied MAPKs are ERK1/2, JNKs, and p38 kinases(Coulombe and Meloche 2007). It has recently been reported that RANKL-mediated activation of three kinases play an important role in osteoclast differentiation(Lee et al. 2009; David et al. 2002; Li et al. 2002). Our data suggested that DHCA specifically suppressed RANKL-induced p38 MAPK pathway, but not the other two pathways involving ERK1/2 and JNK, indicating that DHCA might interact with upstream molecules of the p38 pathway, for

example MAPKK kinases (MAPKKKs) such as MEKKs 1 to 4, MLK2 and -3, and Tak1, or MAPK kinases (MAPKKs) like MEK3 and MEK6(Hommes, Peppelenbosch, and van Deventer 2003; Roux and Blenis 2004). It is also possible that DHCA directly interacts with p38 as in the case of SB203580, a pyridinyl imidazole compound binding to the ATP binding pocket of p38, to regulate its phosphorylation status(Young et al. 1997).

The fact that DHCA activates AMPK is important for several reasons. First, activation of AMPK has been shown to activate RUNX2, a master regulator in the process of osteoblastogenesis(Jang et al. 2011). Since DHCA can also down-regulate the expression of various genes involved in osteoclastogenesis, DHCA may be a very effective agent that can suppress bone loss by controlling both bone resorption and bone formation. Second, DHCA may be a more specific regulator as compared with other plant-derived compounds known to activate AMPK such as resveratrol, curcumin and catechin(He et al. 2010; Kim et al. 2011; Oka et al. 2012). These molecules have been reported to activate all three MAPK pathways including ERK1/2, JNKs, and p38 kinases(Klinge et al. 2005; Syed Hussein, Kamarudin, and Kadir 2015). However, DHCA affected only p38 kinase, but not two other pathways, and thus might have fewer side effects than other plant-derived compounds.

It has been previously reported that estradiol has anti-osteoclastogenic activity via activation of AMPK(Nakamura et al. 2007). Based on this result, we performed experiments to determine whether anti-osteoclastogenic effects of DHCA are mediated through binding to the estrogen receptor. Data from experiments involving siRNAs specific for ERα and ERβ indicated that the effects of DHCA were mediated by ERα, not ERβ. Interestingly, molecular docking simulation performed between DHCA and estrogen receptors predicted that DHCA might bind efficiently to ERα and ERβ. Consistent with this prediction, estrogen receptor competition assay indeed revealed that DHCA might act as a potent agonist for both ERα and ERβ, probably with a relatively small difference in binding affinity. One possible explanation is the difference in tissue distribution of these two estrogen receptors(Bottner, Thelen, and Jarry 2014). ERα is known to be expressed at pre-osteoclast stages, whereas ERβ is expressed at all stages(Krum et al. 2008). Therefore, the anti-osteoclastogenic effects of DHCA might have been mediated

mainly by ERα. If this is indeed the case, DHCA may produce different effects in other tissues or cell types.

Our data from TRAP-staining analysis showed that DHCA affected the size of osteoclasts rather than their number. This may have important implications as cell-cell fusion in osteoclasts is considered to play a critical role in osteoclasts functions through re-organization of the cytoskeleton(Miyamoto 2011). Among three key players (DC-STAMP(Yagi et al. 2005), OC-STAMP(Yang et al. 2008) and P2X7 receptors(Pellegatti et al. 2011)) known to be involved in cell-cell fusion during osteoclastogenesis, DC-STAMP, at least, seems to be a candidate cellular target of DHCA, because the RNA level of DC-STAMP was reduced in a dose-dependent manner when RAW264.7 cells were treated with DHCA. It remains to be elucidated whether DHCA can also influence the expressions of two other genes.

Thus far, hormone replacement therapy involving synthetic 17β-estradiol or conjugated equine estrogens has been commonly used to treat postmenopausal osteoporosis(Shintani 2002). However, there has been safety concerns since the use of such synthetic estrogens might increase the risk of hormone-dependent cancers and cardiovascular diseases(Moreira et al. 2014). In this regard, phytoestrogens have been explored as a possible alternative to synthetic estrogens(Moreira et al. 2014). Many well-known phytoestrogens show a higher affinity for ERβ whereas synthetic estrogens more preferentially bind to ERα(Sunita and Pattanayak 2011). It has been argued that phytoestrogens may be safer than synthetic estrogens as ERβ signaling inhibits mammalian cell growth(Lin et al. 2007). Furthermore, it has been suggested that phytoestrogens may provide other safety benefits thanks to their anti-oxidant activities independent of ER(Patisaul and Jefferson 2010). DHCA seems to bind to both ERα and ERβ with a similar degree of affinity while generating potent anti-oxidant activities via up-regulation of HO-1(Lee and Kim 2014). It remains to be seen whether such characteristics of DHCA could act as a positive or negative factor in developing this lignan molecule as a therapeutic agent for osteoporosis. Given the effective suppression of bone loss and osteoclastogenesis by DHCA, far more extensive in vitro and in vivo characterizations of the phytoestrogen molecule are warranted.

Chapter V

Effects of DHCA on BMP-2-Induced Osteoblastogenesis and Osteoporosis Mouse Model

1. Background

Postmenopausal osteoporosis is a skeletal disease characterized by weakened bone strength and reduced bone mineral density (Kanis 2002; Watts 1999). Estrogen deficiency results in the imbalance between the levels of bone-resorping osteoclasts and bone-forming osteoblasts, eventually leading to overall bone loss (Riggs 2000; Weitzmann and Pacifici 2006). Bone resorption inhibitors such as bisphosphonates are widely used to treat osteoporosis, but their long-term use is known to generate side effects such as severe musculoskeletal pain and hypocalcemia (Kennel and Drake 2009). Therefore, there is a significant unmet medical need for the development of alternative treatment methods with fewer side effects for managing osteoporosis.

Bone formation is mediated by osteoblasts, which is a terminally differentiated form of mesenchymal stem cells (Heino and Hentunen 2008). Osteoblastogenesis, namely the differentiation process of osteoblasts from precursor cells, is regulated by endocrine and paracrine factors such as estrogen and growth factors that activate a variety of intracellular signaling pathways (Nicks et al. 2009) . In osteoblastogenesis, BMP-2 plays a master role in the regulation of various genes involved in osteoblast functions such as RUNX2, ALP, osteocalcin and OPG, by activating the Smad signaling pathway (Lee et al. 2003; Rawadi et al. 2003; Sato et al. 2009; Holtzhausen et al. 2014). The AMPK signaling pathway has also been shown to play an important role(s). Indeed, AMPK activators such as AICAR and metformin has been shown to increase bone nodule formation *in vitro* (Shah et al. 2010).

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from the water-soluble extracts of *Cucurbita moschata* (Lee et al. 2012). DHCA was originally found to contain anti-adipogenic (Lee et al. 2012), anti-inflammatory (Lee, Choi, and Kim 2015) and anti-oxidative stress activities (Lee and Kim 2014). More recently, DHCA has been shown to bind to the estrogen receptor, and similar to estrogen, this lignan molecule did indeed exert anti-osteoclastogenic effects by acting as an estrogen receptor agonist (submitted for publication).

Based on these observations, it was hypothesized that DHCA might promote osteoblastogenesis through its estrogenic activities. In this study, we investigated the effects of DHCA on BMP-2-induced osteoblastogenesis at molecular levels *in vitro*, and on ovariectomy-induced bone loss *in vivo*.

2. Results

2.1 DHCA promotes BMP-2-induced osteoblastogenesis with no cytotoxic effect

MC3T3-E1 is a murine pre-osteoblast cell line that can differentiate into osteoblasts when stimulated with BMP-2 (Ogasawara et al. 2004). To test the effects of DHCA on osteoblastogenesis, MC3T3-E1 cells were treated with BMP-2 (25ng/mL) and three different concentrations of DHCA (10, 20 and 40 μM) for 5 days followed by measuring the number of ALP-positive cells and the activity of ALP. As shown in Figure 18A, the number of ALP-positive cells, as determined by ALP staining, was highly increased by treatment with DHCA in a dose-dependent manner. The effect of DHCA on actual ALP activity was also measured using cellular extracts and pNPP as a substrate. The level of ALP activity was enhanced as DHCA concentration increased (Figure 18B).

The effects of DHCA alone, (that is, in the absence of BMP-2), were also measured. MC3T3-E1 cells were treated with 10, 20, 40 and 80 μM of DHCA. Interestingly, neither the number of ALP-positive cells (Figure 18C) nor the level of ALP activity (Figure 18D) was changed, suggesting that DHCA works only when cells are differentiated by BMP-2.

To be certain, the effects of DHCA on cell viability were measured. MC3T3-E1 cells were cultured with or without BMP-2 in the presence of DHCA followed by MTT assay. As shown in Figure 18E, DHCA had little effect on cell viability throughout all concentrations used in this study, regardless of the presence of BMP-2 during the 72-hour period. Taken together, these data indicated that DHCA might promote the BMP-2-induced osteoblastogenesis without

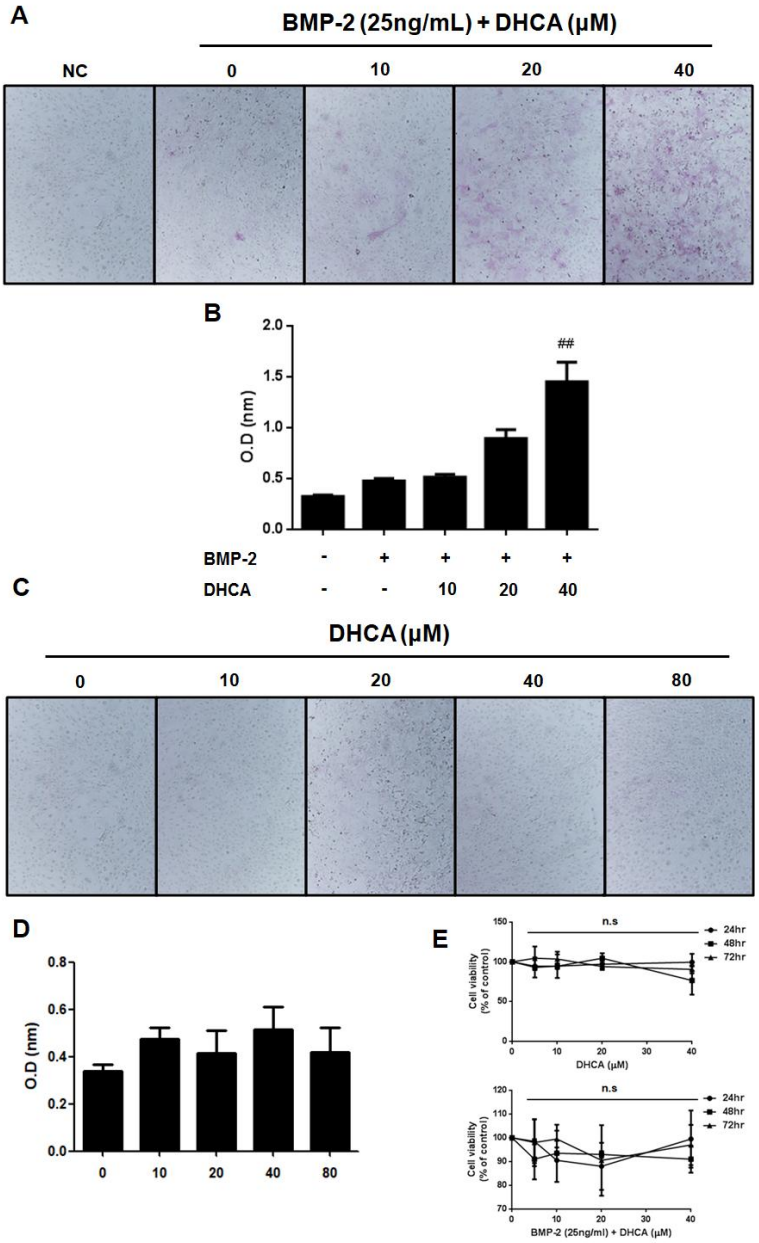

Figure 18. Effects of DHCA on osteoblast differentiation in MC3T3-E1 cells. MC3T3-E1 cells were treated with BMP-2 (25ng/mL) and DHCA (10, 20 and 40 μM) for 5 days. (A) ALP-positive cells were visualized by ALP stain; (B) ALP activity was measured at 450nm following ALP activity assay as described in Materials and Methods. MC3T3-E1 cells were treated with DHCA (10, 20, 40 and 80 μM) for 5 days. (C) ALP-positive cells were visualized by ALP stain; (D) ALP activity was measured at 450nm following ALP activity assay as described in Materials and Methods. (E) MC3T3-E1 cells were treated with or without BMP-2 (25ng/mL) in the presence of different concentrations of DHCA (10, 20 and 40 μM) for 72 hours. Cells were then subjected to MTT assay as described in the Materials and Methods section. Values represent the mean ± S.E.M. of three independent experiments. $^{\#\#}p<0.01$ compared with that treated with BMP-2 alone.

cytotoxic effects.

2.2 DHCA regulated the expression of genes associated with osteoblast differentiation

It has previously been reported that stimulation of MC3T3-E1 cells by BMP-2 up-regulates the expression of ALP, osteocalcin and OPG, which all play important roles in the differentiation and function of osteoblasts (Jang et al. 2012; Rawadi et al. 2003; Sato et al. 2009). To study the effects of DHCA on the expression of these genes, MC3T3-E1 cells were treated with BMP-2 (25ng/mL) and DHCA (10, 20 and 40 μM) for 24 hours, and the RNA level was determined by quantitative RT-PCR. In all three cases, BMP-2 treatment increased their RNA levels by 2-3 fold. When cells were co-treated with 40μM of DHCA, their levels were further enhanced by 2 fold in a dose-dependent manner (Figure 19).

2.3 DHCA promoted BMP-2-induced RUNX2 production via Smad and AMPK activation

During BMP-2-induced osteoblastogenesis, RUNX2 becomes activated by the Smad signaling pathway. This is a critical step in the differentiation of osteoblasts (Phimphilai et al. 2006), while AMPK has also been shown to play a positive role in this process (Jang et al. 2011). To test the effects of DHCA on the BMP-2-induced expression of RUNX2, MC3T3-E1 cells were co-treated with BMP-2 and three different concentrations of DHCA (10, 20 and 40 μM) for 24 hours, and the protein level of RUNX2 was measured by Western blot. When cells were treated with BMP-2, the protein level of RUNX2 was increased (Figure 20A, compare lanes 1 and 2), and co-treatment with DHCA further enhanced the protein level of RUNX2 (Figure 20A, compare lanes 2 and 5).

We also measured the effects of DHCA on other signaling proteins involved in the

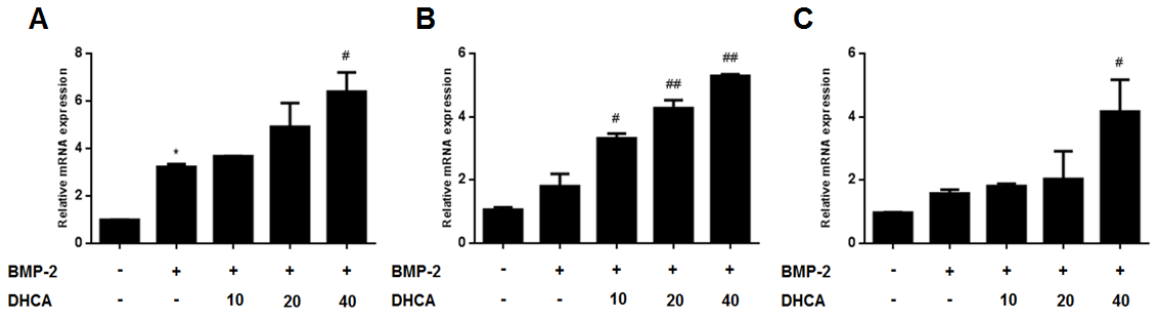

Figure 19. Effects of DHCA on the expression of osteoblastogenic genes during BMP-2-induced osteoblastogenesis. MC3T3-E1 cells were treated with BMP-2 (25ng/mL) and cultured in the presence of DHCA (10, 20 and 40 μM) for 24 hours. Total RNAs were isolated and analyzed by quantitative RT-PCR for ALP (A), Osteocalcin (B) and OPG (C). Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$ compared with control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with that treated with BMP-2 alone.

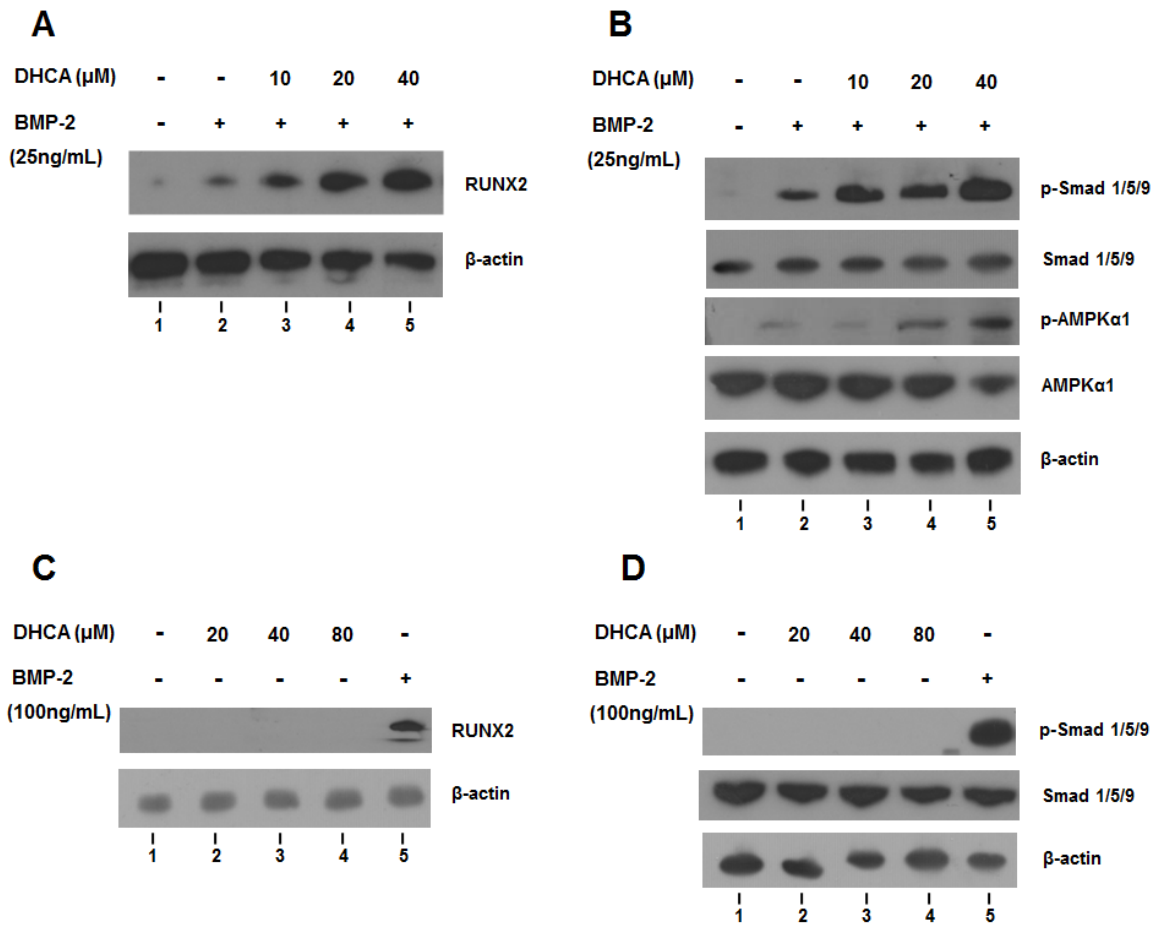

Figure 20. Effects of DHCA on osteoblastogenesis-related signaling pathways. MC3T3-E1 cells were treated with BMP-2 (25ng/mL), and cultured in the presence of DHCA (10, 20 and 40 μM) for 2 hours or 30 min for RUNX2 or Smad1/5/9 and AMPKα1, respectively. Total protein were prepared following by western blot using antibodies specific for respective proteins; (A) Effects of DHCA on BMP-2-mediated increase of RUNX2; (B) Effects of DHCA on BMP-2-mediated activation of Smad1/5/9 and AMPKα1. MC3T3-E1 cells were treated with DHCA (20, 40 and 80 μM) or BMP-2 (100ng/mL) as a positive control for 2 hours or 30 min for RUNX2 or Smad1/5/9, respectively. Total protein were prepared following by western blot using antibodies specific for respective proteins; (C) Effects of DHCA on BMP-2-mediated increase of RUNX2; (D) Effects of DHCA on BMP-2-mediated activation of Smad1/5/9.

BMP-2-induced signaling pathway. MC3T3-E1 cells were co-treated with BMP-2 and DHCA for 30 minutes, and the phosphorylation status of Smad1/5/9 and AMPK were each determined by Western blot. When cells were treated with BMP-2, the level of phosphorylated Smad1/5/9 was highly increased (Figure 20B, compare lanes 1 and 2), and co-treatment with DHCA further enhanced the amount of this phosphorylated protein (Figure 20B, compare lanes 2 and 5). Similarly, phosphorylation of AMPK was also up-regulated by DHCA treatment (Figure 20B). However, DHCA alone, namely in the absence of BMP-2, did not have any effect on the level of RUNX2 and phosphorylated Smad1/5/9 (Figure 20C and D). These data indicated that DHCA could up-regulate the RUNX2-related signaling pathways, but only when cells were already in an activated status by BMP-2.

2.4 Effects of DHCA on osteoblastogenesis were mediated by ERα and ERβ

It is well known that estrogen promotes early osteoblast differentiation (Okazaki et al. 2002). To test the effects of DHCA on the estrogen-induced signaling pathway, MC3T3-E1 cells were transfected with a luciferase reporter plasmid containing the nucleotide sequences for estrogen responsive element (ERE). Twenty-four hours later, transfected cells were treated with estradiol or DHCA for 6 hours. Total proteins were extracted, and the relative level of luciferase activity was measured. When cells were treated with DHCA, the level of luciferase activity was increased in a dose-dependent manner (Figure 21A), by 2.6-fold at 40μM, indicating that DHCA might interact with the estrogen receptor in MC3T3-E1 cells.

There are two different types of estrogen receptors, ERα and ERβ, and each has different functions due to its difference in their affinity for ligands (Dechering, Boersma, and Mosselman 2000; Lee, Kim, and Choi 2012). To investigate which of the two estrogen receptors interacts with DHCA to exert the observed effects, MC3T3-E1 cells were transfected with siRNA against ERα or ERβ followed by treatment with BMP-2 and DHCA. First, specificity of siRNA was measured. Cells were transfected with 30 pmole of siRNA for each receptor, and the protein level of ERα and ERβ was measured by Western blot. In both cases, the protein level

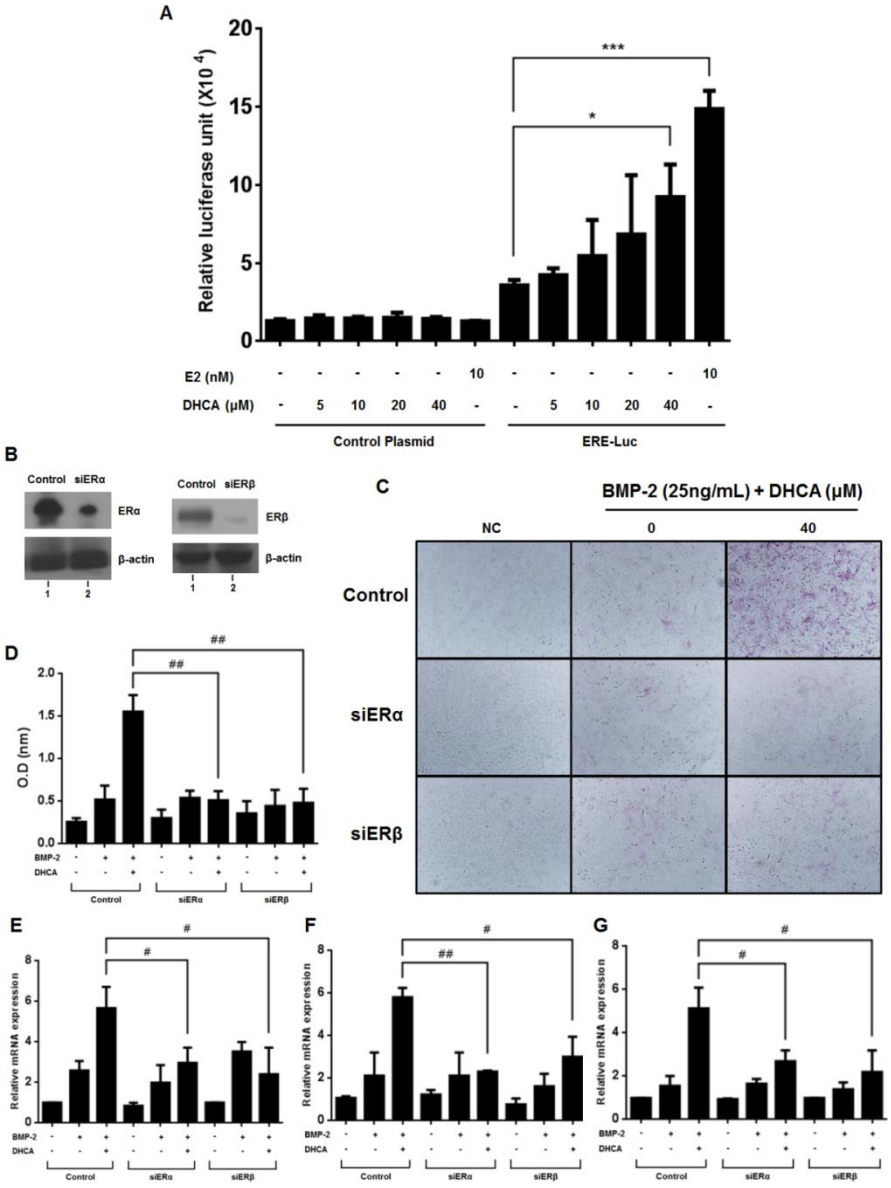

Figure 21. Effects of DHCA on the estrogen receptors during BMP-2-induced osteoblastogenesis. MC3T3-E1 cells were transfected with control or luciferase reporter plasmid containing sequences for ERE, then were treated with DHCA (5, 10, 20 and 40 μM) or E2 (10nM) as a positive control for 6 hours. (A) Luciferase activity was measured. MC3T3-E1 cells were transfected with siRNA against either ERα, ERβ or scramble control, then were co-treated with BMP-2 (25ng/mL) and DHCA (40uM). (B) ERα and ERβ protein were knocked down by siRNA; (C) ALP-positive cells were visualized by ALP stain after 5 days; (D) ALP activity was measured at 450nm following ALP activity assay after 5 days as described in Materials and Methods; The RNA level of ALP (E), Osteocalcin (F) and OPG (G) was analyzed by quantitative RT-PCR after 24 hours. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with control siRNA group that treated with BMP-2 and DHCA.

was highly reduced (Figure 21B). Next, the effect of siRNAs on the osteoblastogenesis was determined by measuring the number of ALP-positive cells and the level of ALP activity when cells were treated with BMP-2 (25ng/mL) and DHCA (40 μM). As shown in Figure 21C and D, both parameters were highly decreased when cells were transfected with siRNAs for ERα or ERβ. The effect of siRNAs was also measured on the BMP-2/DHCA-mediated activation of three osteoblastogenic genes (ALP, osteocalcin and OPG), and the RNA levels of all three genes were highly reduced (Figure 21E-G). Taken together, these data indicated that DHCA might interact with both ERα and ERβ to promote BMP-2-induced osteoblast differentiation.

2.5 DHCA blocks Ovx-induced bone loss and decreases osteoclastogenic gene expression in bone marrow.

The above data shows the potential therapeutic effects of DHCA in osteoporosis. To test this possibility, ovariectomized-mice model was used in which the ovary is physically removed, resulting in phenotypes similar to osteoporosis(Jee and Yao 2001). The ovary was removed on day 0, and various concentrations of DHCA were injected intraperitoneally on a daily basis to ovariectomized mice, and femurs were analyzed by micro-CT after 4 weeks. As shown in Figure 22A and B, ovariectomized mice showed a significant bone loss, and this effect was diminished upon DHCA treatment in a dose-dependent manner. The 3D-structure analysis showed that the removal of the ovary reduced various bone morphometric parameters such as trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecularnumber (Tb.N), and bone marrow density (BMD), as compared to the control. Effects of DHCA treatment were visually clear in all these parameters (Figure 22C). Similarly, trabecular bone surface (BS/BV), trabecular spacing (Tb.Sp), trabecular pattern factor (Tb.Pf), and structure model index (SMI) were increased in the sham group, and these effects were diminished with DHCA treatment with the exception SMI (Figure 22C).

The blood level of N- or C- telopeptide of type I collagen (NTx or CTx)

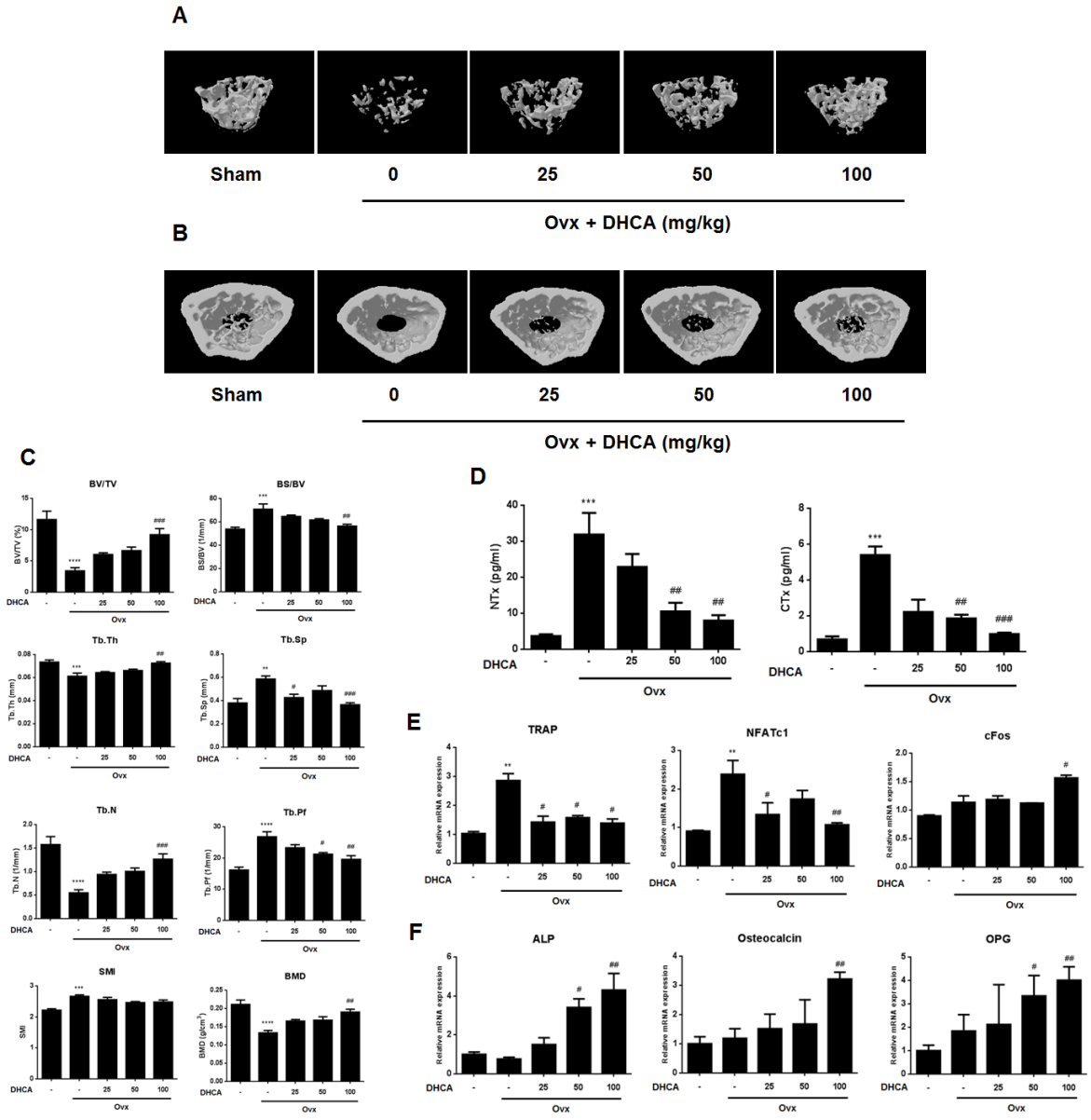

Figure 22. Effects of DHCA on ovariectomy-induced bone loss. (A) 3D-structure of trabecular bone in proximal femur were obtained by the μCT; (B) 2D-image of proximal femur were obtained by the μCT; (C) bone volume over total volume (BV/TV, %), bone surface density (BS/BV, 1/mm), trabecular thickness (Tb.Th, mm), trabecular spacing (Tb.Sp, mm), trabecular number (TB.N, 1/mm), trabecular pattern factor (TB.Pf, 1/mm), structure model index (SMI), and bone mineral density (BMD, g/cm^3) were obtained by the μCT; (D) Serum NTx and CTx was measured by ELISA; (E) Osteoclastogenic and (F) Osteoblastogenic gene expression in bone marrow were measured by quantitative RT-PCR; 7-wk Balb/c mice were subjected to sham operation or OVX, then vehicle or various concentrations of DHCA was injected i.p for 4 weeks. Values represent the mean ± S.E.M. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ compared with control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with Ovx group.

(Hankenson et al. 2005; Katsumata et al. 2015), a biochemical marker for bone degradation, was also measured by ELISA. DHCA decreased the blood level of NTx and CTx which had been elevated by Ovx (Figure 22D).

Finally, osteoclastogenic and osteoblastogenic gene expression patterns were also analyzed by measuring the RNA level of TRAP, NFATc1, c-Fos, ALP, osteocalcin and OPG in bone marrow by quantitative RT-PCR. In the sham group, the RNA level of osteoclastogenic genes was increased, while it was significantly lowered in DHCA-treated animals (Figure 22E). Furthermore, osteoblastogenic gene expressions were promoted when animals were treated with DHCA (Figure 22F). Taken together, these data indicated that DHCA might inhibit bone loss mediated by estrogen deficiency.

2.6 DHCA was predicted to bind with ERα and ERβ by molecular docking simulation.

Our data from Figure 17 and 21 suggested that DHCA may bind with estrogen receptor. To study this possibility, molecular docking simulation was performed. The structures of ERα and ERβ ligand binding domain from *mus musculus* were built using human ER structure by homology modeling, and potential binding poses with estradiol or DHCA were generated using Glide software(Shen et al. 2013). As shown in Figure 23A and B, DHCA was estimated to show a similar binding pose with ERα. Furthermore, the 2D ligand-receptor interaction diagram showed that intermolecular forces, such as hydrogen bonds and π-π interactions between ERα and DHCA, were analogous to the case of estradiol (Figure 23C and D). These data indicated that DHCA might bind to ERα. The same analysis predicted that DHCA might also interact with ERβ (Figure 23E–H). Consistent with these analyses, DHCA was predicted to have sufficient MM-GBSA binding energy to bind with ERα and ERβ (Table 3). Taken together, these data indicated that DHCA might have the potential to bind to the estrogen receptor family, but that, the biological outcome might be different depending on cellular targets.

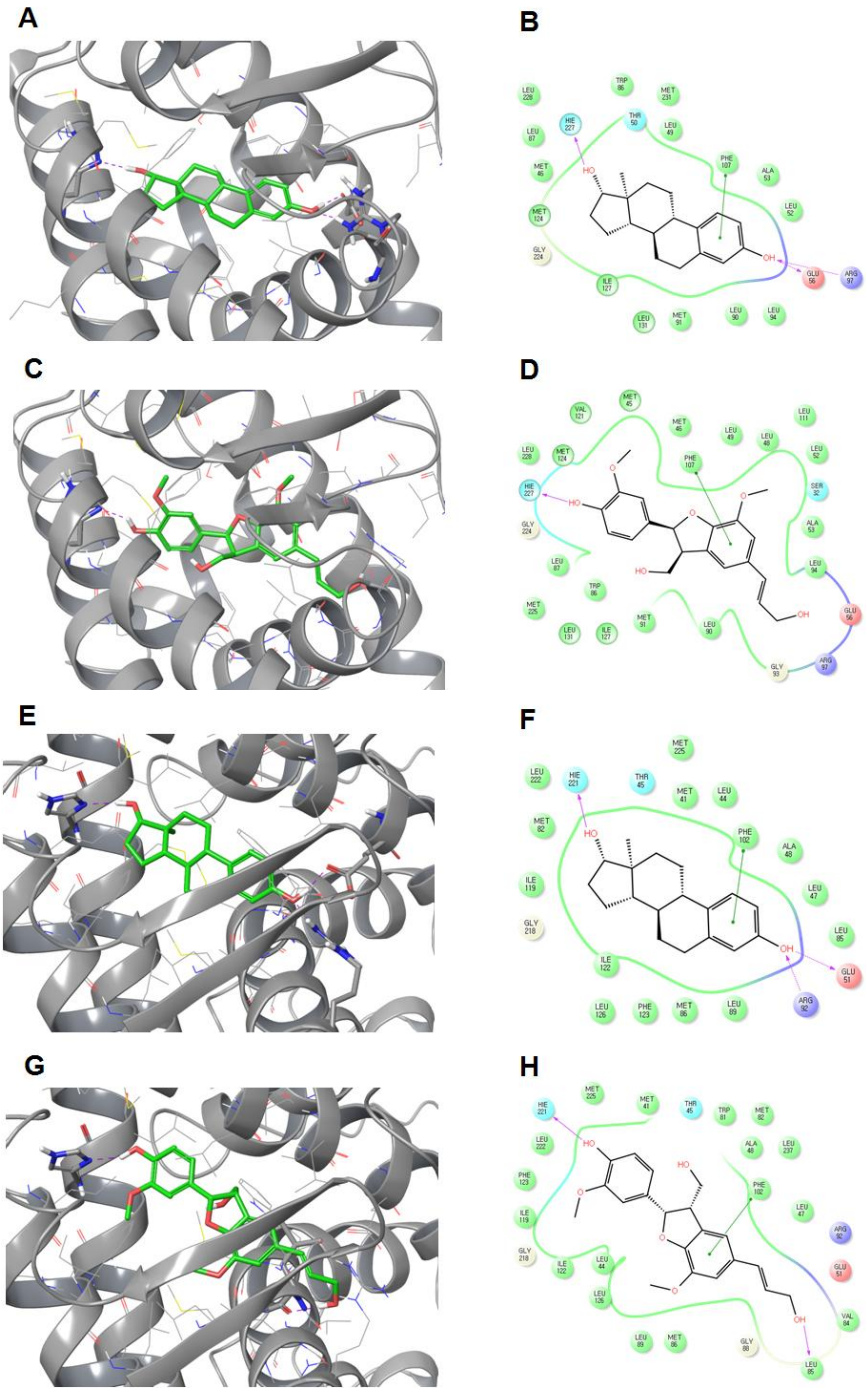

Figure 23. DHCA was predicted to bind with ERα and ERβ by molecular docking simulation. (A) Crystal structure of ERα LBD in complex with 17β-estradiol; (B) 2D ERα-17β-estradiol interaction diagram. Hydrogen bonds (purple arrow) and π-π interactions (green arrow) are shown; (C) Crystal structure of ERα LBD in complex with DHCA; (D) 2D ERα-DHCA interaction diagram; (E) Crystal structure of ERβ LBD in complex with 17β-estradiol; (F) 2D ERβ-17β-estradiol interaction diagram; (G) Crystal structure of ERβ LBD in complex with DHCA; (H) 2D ERβ-DHCA interaction diagram. Ligand-receptor molecular docking was simulated by Glide (see Materials and Methods).

Receptor	Ligand	MM-GBSA binding energy (Kcal/mol)
ERα	DHCA	-82.449
	Estradiol	-106.576
ERβ	DHCA	-83.465
	Estradiol	-112.859

Table 3. MM-GBSA binding energy

2.7 DHCA acts as agonist on both estrogen receptor alpha and beta.

To determine whether DHCA binds to the ERα and ERβ, estrogen receptor competition assay was performed. Various concentrations of DHCA were added to estrogen receptor / fluormone tracer complex for 2 hours, and the fluorescence polarization value was measured. As shown in Figure 24A and B, DHCA bound to ERα and ERβ. The IC_{50} values obtained from these experiments indicate that DHCA binds both ERα and ERβ with lower affinity than estradiol. The estimated IC_{50} values for ERα and ERβ were 61.91 and 286.5 nM respectively (Table 4). Therefore, DHCA acts as ERα agonist that displays 4.63-fold selectivity over ERβ, however the difference in IC_{50} between ERα and ERβ was not significant. These data indicated that DHCA might show the estrogenic effects through binding to both ERα and ERβ.

3. Discussion

DHCA is a lignan compound isolated from the water-soluble extracts of *Cucurbita moschata* (Lee et al. 2012). It was previously shown to contain anti-adipogenic activities in 3T3-E1 cells, and also anti-inflammatory (Lee, Choi, and Kim 2015) and anti-oxidative activities (Lee and Kim 2014) in macrophage and lymphocyte cell types. In this study, we investigated the effects of DHCA on BMP-2-induced osteoblastogenesis using the MC3T3-E1 pre-osteoblast cell line. DHCA increased the number of ALP-positive cells as well as the level of ALP activity. This lignan molecule further up-regulated the BMP-2 mediated activation of Smad1/5/9 and AMPK signaling pathways, involving the expression of RUNX2 and subsequently that of genes such as ALP, osteocalcin and OPG. These osteoblastogenic effects of DHCA were attenuated by inhibiting both ERα and ERβ using specific siRNAs. Consistent with these in vitro data, DHCA could effectively suppress bone loss and osteoclastogenesis induced by ovariectomy in the mouse model. The results from molecular docking simulation and receptor competition assays indicated that DHCA might act as an agonist on both ERα and ERβ.

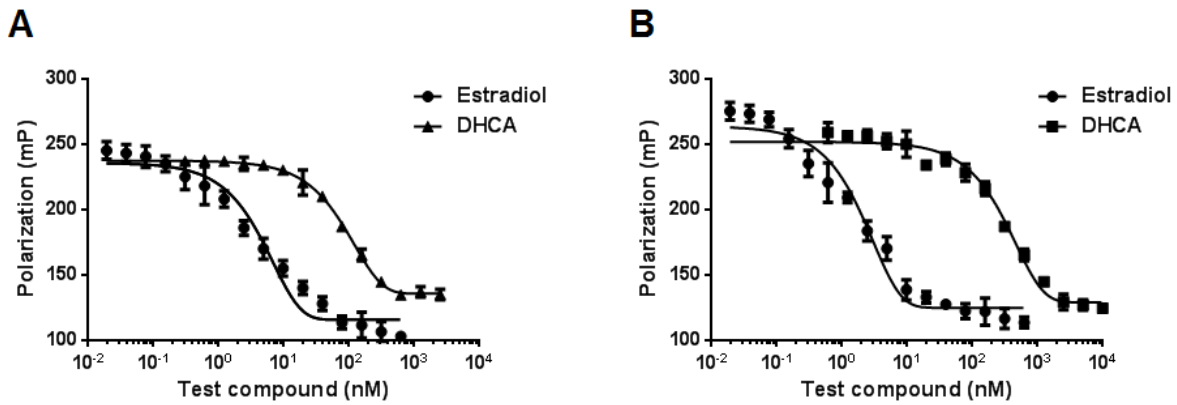

Figure 24. DHCA acts as agonist on both ERα and ERβ. (A) Polarization values for ERα against the concentration of DHCA; (B) Polarization values for ERβ against the concentration of DHCA. Estrogen receptor competition assay was performed using PolarScreen™ ERα / β competitor assay kit (see Materials and Methods).

Receptor	Test compound	IC_{50} (nM)
ERα	Estradiol	4.804
	DHCA	61.91
ERβ	Estradiol	1.280
	DHCA	286.5

Table 4. IC50 values

DHCA alone, that is, in the absence of BMP-2, did not affect either osteoblast differentiation and RUNX2-related Smad signaling pathway. The fact that DHCA does not work when cells contain unactivated, null Smad 1/5/9, means that DHCA exerts its effects by regulating another molecule(s) involved in the control of BMP-2 mediated activation. One possible explanation is that DHCA affects PPM1H, which is a Smad1/5/9-specific phosphatase (Shen et al. 2014). It has been shown that the ectopic expression of PPM1H inhibits BMP signaling, while suppression of PPM1H by siRNA promotes the expression of those genes controlled by BMP-2 and enhances osteoblast differentiation (Shen et al. 2014). Therefore, it may be possible that DHCA regulates osteoblast differentiation through the inhibition of the PPM1H-related actions. To our knowledge, there has been no report on the relationship between estrogen and PPM1H. It remains to be seen whether DHCA indeed controls PPM1H.

We previously reported that DHCA could suppress the expression of PPARγ, inhibiting adipocyte differentiation (Lee et al. 2012). Peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor activated by long chain fatty acid or peroxisome proliferators which play a key role(s) in adipocyte differentiation (Siersbaek, Nielsen, and Mandrup 2010). PPARγ was recently found to be involved in osteoblast differentiation (Takeda and Takeuchi 2004). For example, when mice were treated with PPARγ activator, TZD, significant bone loss was observed due to an increase in marrow adipocytes together with a decrease in osteoblasts, while in PPARγ deficient mice, bone formation and osteoblastogenesis were enhanced (Viccica, Francucci, and Marcocci 2010). Therefore, it may be possible that DHCA controls osteoblast differentiation through PPARγ-related actions as well as estrogen receptor-mediated actions.

Wnt/β-catenin signaling is well known to play important roles in embryonic and postnatal developmental processes (Dravid et al. 2005; Hai et al. 2010). It has been recently shown that Wnt/β-catenin signaling controls osteoblast differentiation by cross-talking with BMP-2 signaling (Zhang et al. 2013). Treatment with Wnt-ligand, Wnt3a, increased the level of luciferase activity from the BMP/Smad reporter plasmid, and also the expression of osteoblastogenic genes and the level of ALP activity in C2C12 cells (Zhang et al. 2013).

Furthermore, co-treatment with Wnt3a and BMP-2 further increased the level of osteoblastogenic gene expression and ALP activity compared to when either activator was used (Zhang et al. 2013). Estrogen receptor signaling facilitates osteogenic differentiation by cross-talking with Wnt/β-catenin signaling (Gao et al. 2013), while in this study, DHCA was revealed as an agonist for estrogen receptor. Taken together, DHCA may up-regulate BMP-2 induced osteoblastogenesis by affecting the Wnt/β-catenin signaling pathway.

Our data indicate that DHCA promotes BMP-2-induced osteoblast differentiation by interacting with either ERα or ERβ as an agonist for both receptors. We have recently shown that DHCA inhibits RANKL-induced osteoclast differentiation in vitro and ovariectomy-induced bone loss in vivo (submitted for publication). Taken together, our results indicate that DHCA may be developed as an efficient therapeutic for osteoporosis by controlling the osteoclast/osteoblast ratio through its estrogenic effects.

Chapter VI

Concluding Remarks

DHCA was found from stem parts of *Cucurbita moschata* in our laboratory around the year 2008 (and published in 2012) during the search for anti-obesity bioactivities from plant sources(Lee et al. 2012). At that time, various extracts and fractions were prepared from green pumpkin, a common name of *C. moschata*, and tested on their effects on differentiation of 3T3-E1 cells to adipocytes when stimulated by adipokines. When the structure of the compound contained in the fraction showing the highest activity was determined, it was DHCA(Lee et al. 2012). Since then, DHCA has been intensively studied in our laboratory. In previous studies performed in our laboratory, DHCA has been shown to contain anti-adipogenic, anti-oxidative stress(Lee and Kim 2014) and anti-inflammatory activities(Lee, Choi, and Kim 2015), mostly using in vitro cell culture systems. For my thesis work, I explored the possibility of using DHCA for specific diseases, psoriasis and osteoporosis, by testing anti-inflammatory, anti-osteoclastogenic and osteoblastogenic effects of this lignan molecule and then studying the underlying molecular mechanisms, in vivo as well as in vitro.

DHCA showed strong anti-inflammatory effects against IL-17 mediated inflammatory responses in HaCaT keratinocyte cell line. It effectively inhibited the IL-17-mediated cell proliferation without any cytotoxic effects. Moreover, DHCA also suppressed the expression of various inflammatory mediators, such as TNF-α, IL-6, IL-1β, CXCL1, CXCL8, CCL20, S100A8, S100A9 and LL-37, through the specific inhibition of p38 MAPK signaling pathway.

In imiquimod-induced psoriasis-like skin inflammation mouse model, DHCA ameliorated the psoriatic symptoms, such as erythema, scaling, thickening and histological phenotypes including hyperkeratosis and elongation of rete-like ridge, and lowered the expression of various inflammatory mediators in a dose-dependent manner. Data from immunohistochemical analysis and ex vivo culture experiment also suggested that DHCA reduced the infiltration of IL-17 producing inflammatory cells such as neutrophils and T cells into the back skin. These inhibitory effects of DHCA on immune cell infiltrations were found to

be mediated by suppressing various chemokines such as CXCL1, CXCL8 and CCL20 at early-time points.

Being a lignan molecule, DHCA is classified to be a member of the phytoestrogen family, and thus may contain estrogenic effects. Therefore, I investigated whether DHCA regulates the ratio of osteoclast to osteoblast, using relevant cell lines, and attempted to unravel the detailed molecular mechanism underlying its effects. In RAW264.7 pre-osteoclast cell line, DHCA effectively inhibited the RANKL-induced differentiation and function of osteoclast without any cytotoxic effects in a dose-dependent manner. DHCA also suppressed the expression of various osteoclastogenic genes, such as NFATc1, TRAP, c-Fos, DC-STAMP, MMP-9, and cathepsin K, through the inhibition of the Akt-IKK-NF-κB signaling pathway and the p38 MAPK signaling pathway. Furthermore, DHCA increased the level of phosphorylated AMPK, while the above anti-osteoclastogenic effects of DHCA were diminished when AMPKα1 expression was knocked down with siRNA. These effects of DHCA were suppressed when cells were transfected with ERα siRNA, whereas these effects were not affected by transfection with ERβ siRNA.

DHCA also promoted the BMP-2-induced differentiation of osteoblast without any cytotoxic effects in MC3T3-E1 pre-osteoblast cell line. This lignan molecule further up-regulated the BMP-2 mediated activation of Smad1/5/9 and AMPK signaling pathways, the expression of RUNX2 and subsequently that of genes such as ALP, osteocalcin and OPG. Interestingly, these osteoblastogenic effects of DHCA were not observed in the absence of BMP-2. Gene knockdown analysis using ERα or ERβ specific siRNA indicated that DHCA might interact with both ERα and ERβ to promote the BMP-2-induced osteoblast differentiation.

These data indicated that DHCA might produce anti-osteoporotic activities by regulating the differentiation of osteoclast and osteoblast through its agonistic effect on estrogen receptor. Therefore, it was tested whether DHCA could improve the osteoporotic phenotypes in

the ovariectomized mouse model. Data from 3D-structure analysis showed that the removal of the ovary reduced various bone morphometric parameters such as BV/TV, Tb.Th, Tb.N, and BMD, as compared to the control, and treatment with DHCA efficiently improved all these parameters. Similarly, the levels of BS/BV, Tb.Sp, Tb.Pf, and SMI were increased in the sham group, and all these effects except on SML were diminished when DHCA was used. Treatment with DHCA also lowered the blood level of NTx and CTx, biochemical markers for bone degradation. Furthermore, DHCA down-regulated the expression of osteoclastogenic genes such as TRAP and NFATc1, while up-regulating the expression of osteoblastogenic genes such as ALP, osteocalcin and OPG in the bone marrow.

Data from Figure 17 and 21 showed that DHCA might act as an estrogen receptor agonist. In addition, a variety of biological activities previously shown in our laboratory, including anti-oxidative stress, anti-inflammatory, anti-osteoclastogenic and osteoblastogenic activities, can be all explained if DHCA acts like estrogen. Therefore, I investigated whether the DHCA was an agonist of the estrogen receptor. Data from molecular docking simulation suggested that DHCA was estimated to bind to both ERα and ERβ with a similar binding pose to estrogen. Furthermore, the 2D ligand-receptor interaction diagram showed that intermolecular forces, such as hydrogen bonds and π-π interactions between estrogen receptors and DHCA, are analogous to the case of estradiol. DHCA was also predicted to have sufficient MM-GBSA binding energy to bind with both ERα and ERβ. Consistent with these analyses, data from estrogen receptor competition assay showed that DHCA efficiently bound to ERα and ERβ. The estimated IC50 values for ERα and ERβ were 61.91 and 286.5 nM, respectively.

Together with previously published data, DHCA appears to work as summarized in Figure 25. DHCA enters the cell, presumably by diffusion, interacts with the estrogen receptor in the cytoplasm, and phosphorylates AMPK and Smad, resulting in the inhibition of NFATc1

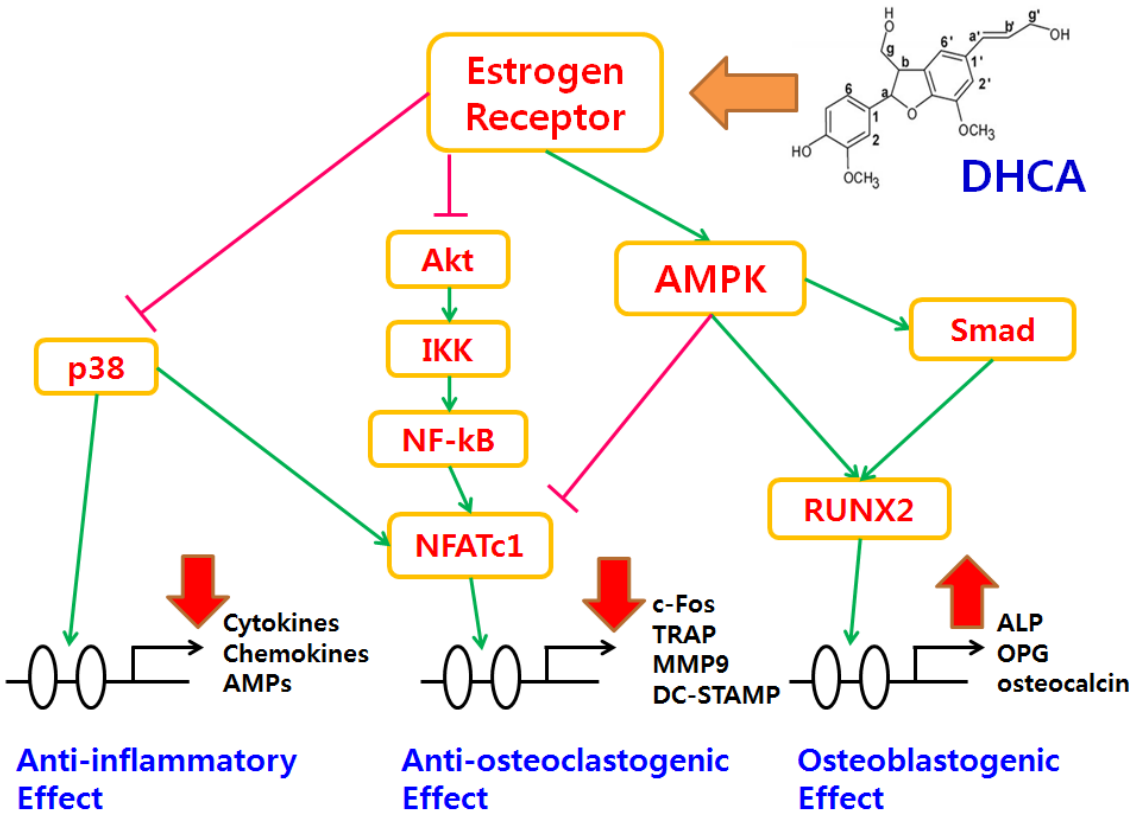

Figure 25. Schematic diagram of mechanism(s) of DHCA. A wide range of proteins are involved in the regulation of various cellular responses such as inflammation, osteoclastogenesis and osteoblastogenesis. The effects of DHCA on these signaling pathways are mediated by estrogen receptor.

and RUNX2 activities, and subsequently exerting anti-osteoclastogenic and osteoblastogenic effects. Independently, the interaction between DHCA and the receptor inhibits the phosphorylation of p38, leading to the suppression of production of a variety of inflammatory molecules, including cytokines and chemokines, thereby producing potent anti-inflammatory effects.

DHCA is very safe in the high dose and repeated dose experiments performed previously in our laboratory (unpublished results), while containing multiple bioactivities that can be used for a variety of human diseases, including psoriasis and osteoporosis as shown by my thesis work. Further studies are warranted to investigate the possibility of clinical applications of DHCA and to perform necessary experiments such as pharmacokinetics, pharmacodynamics and optimization of chemical structure among others.

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국문 초록

Dehydrodiconiferyl alcohol(DHCA)는 원래 *Cucurbita moschata*(애호박)의 줄기에서 분리한 분자량 358 의 리그난 계열 물질이다. 우리 연구실에서는 과거에 합성한 DHCA 가 항비만, 항산화 및 항염증 기능을 가진다고 보고한 바 있다. 본 연구에서는 DHCA 가 보여주는 강력한 항염증 효과를 분자세포생물학적으로 해석하고, 식물성 에스트로겐의 구조를 보이는 DHCA 의 항골다공증 활성에 대해 in vitro 와 in vivo 에서 분석하였다.

염증 연구에 많이 사용되는 HaCaT 표피세포주를 사용한 실험에서, DHCA 는 IL-17 에 의해 매개된 세포의 증식과 각종 염증성 매개체(TNF-α, IL-6, IL-1β, CXCL1, CXCL8, CCL20, S100A8, S100A9 및 LL-37)들의 발현을 농도 의존적으로 저해하였다. 또한 DHCA 는 IL-17 에 의해 활성화된 MAPK 중 p38 의 활성을 특이적으로 억제하였다. 이와 같은 DHCA 의 항염증 활성을 바탕으로 imiquimod 로 유도한 건선 유사 염증 마우스 모델에서 DHCA 의 효능을 조사하였다. DHCA 는 PASI score 와 표피조직의 과증식, 각질의 과생성을 포함하는 건선의 증상, 피부 조직에서의 염증성 매개체의 발현을 농도 의존적으로 저해하였다. 면역조직학적 염색과 ex vivo 배양 실험에서, DHCA 는 IL-17 을 분비하는 세포의 침윤을 감소시킴이 관찰되었는데, 이는 DHCA 에 의해 초기 단계에서 세포의 침윤을 유도하는 chemokine 의 생성이 억제되기 때문인 것으로 생각된다.

DHCA 는 식물 유래 화합물이며 에스트로겐과 유사한 구조를 가지고 있어, 식물성 에스트로겐으로 분류된다. 이에 착안하여 DHCA 가 에스트로겐 유사 효과를 나타내어 항골다공증 활성을 가지는지를 밝히고자 하였다. 우선 RAW264.7 pre-osteoclast 세포주를 사용하여 뼈의 분해를 매개하는 파골세포의 분화에 DHCA 가 미치는 영향을 조사하였다. DHCA 는 RANKL 에 의해 유도된 파골세포로의 분화와

기능 그리고 분화에 관여하는 유전자의 발현을 농도 의존적으로 감소시켰다. 또한 DHCA 는 RANKL 에 의해 유도된 Akt-IKK-NF-kB 신호전달경로와 p38 MAPK 신호전달경로를 억제하였고, 반면 파골세포의 분화 조절에 관여하는 AMPK 신호전달경로는 활성화하였다. 이와 같은 DHCA 의 효과는 에스트로겐 수용체 알파를 억제할 시에는 감소하였으나, 에스트로겐 수용체 베타를 억제할 경우에는 영향을 받지 않았다.

다음으로 DHCA 가 뼈의 생성을 매개하는 조골세포의 분화에 미치는 영향을 분석하였다. MC3T3-E1 pre-osteoblast 세포주에서, DHCA 는 BMP-2 에 의해 유도된 조골세포의 분화와 관계된 유전자의 발현을 농도 의존적으로 증가시켰다. 또한 DHCA 는 BMP-2 에 의해 유도된 Smad 신호전달경로와 AMPK 신호전달경로를 활성화시켰다. 하지만 이러한 효과는 DHCA 단독으로는 나타나지 않았으며, BMP-2 와 동시에 처리할 때만 나타났다. 또한 DHCA 의 조골세포 분화 촉진 효과는 에스트로겐 수용체 알파와 베타를 저해하였을 경우 나타나지 않았다.

이와 같은 실험결과를 바탕으로 DHCA 가 골다공증 질환 모델인 난소절제 마우스 모델에서 항골다공증 효능을 나타내는지 조사하였다. 난소 절제 후 4 주 후에 넓적다리 뼈의 3 차원 구조, 혈액 내의 뼈 분해 바이오마커, 그리고 골수 세포에서 유전자 발현 정도를 평가한 결과, 난소 절제 시 이와 같은 지표들이 모두 유의미하게 증가하였지만, 이와 같은 증상 모두가 DHCA 처리군에서는 현격하게 회복됨을 확인하였다.

이와 같은 실험결과는 DHCA 가 에스트로겐 유사 활성을 나타냄을 의미한다. 또한 기존 보고에 기술된 DHCA 의 항비만, 항산화, 항염증 및 항골다공증 효과 역시, DHCA 가 에스트로겐의 활성을 가진다면 모두 가능한 현상들이다. 이에 착안하여 DHCA 가 실제로 에스트로겐 수용체와 결합할 가능성에 대해 조사하였다. DHCA 와 에스트로겐 수용체 간의 결합 시뮬레이션 결과,

DHCA 는 에스트로겐 수용체 알파와 베타 모두 에스트로겐과 유사한 binding pose 로 결합할 수 있음을 예측할 수 있었다. 또한 에스트로겐 수용체에 대한 경쟁적 결합 실험 결과, DHCA 는 에스트로겐 수용체 알파와 베타 모두에 효과적으로 결합하는 것이 밝혀졌다.

결론적으로 DHCA 는 in vitro 와 in vivo 모두에서 항염증과 항골다공증 활성을 나타내었다. 또한 DHCA 는 에스트로겐 수용체의 agonist 로 작용하여, 다양한 에스트로겐의 활성을 나타낼 수 있음이 밝혀졌다. 식물성 에스트로겐은 부작용이 에스트로겐에 비해 현저히 적음이 알려져 있으며, 이 연구에서 수행된 모든 농도에서도 DHCA 는 세포나 동물에 대한 독성을 나타내지 않았다. 모든 것을 고려할 때 DHCA 는 건선과 골다공증과 같은 여러가지 만성 질환들에 대한 치료제로 개발할 수 있을 것으로 판단된다.

중심어 : Dehydrodiconiferyl alcohol, inflammation, phytoestrogen, osteoclast, osteoblast, IL-17, RANKL, BMP-2, IMQ-induced psoriasis-like skin inflammation model, ovariectomy-induced mouse model, estrogen receptor

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