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Nitric Oxide is a Mediator of Osteoblast Growth and Function

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NITRIC OXIDE IS A MEDIATOR OF OSTEOBLAST GROWTH AND FUNCTION

By

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ABSTRACT

NITRIC OXIDE IS A MEDIATOR OF OSTEOBLAST GROWTH AND FUNCTION

By

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Mechanical strain and sex hormones stimulate osteoblasts to produce low amounts of nitric oxide that may be beneficial for bone growth, remodeling and maintenance. In contrast, higher levels of nitric oxide induced by cytokines can inhibit bone formation and possibly cause cell death at higher non-physiologic concentrations. I hypothesize that levels of NO seen under conditions of mechanical loading stimulate osteoblast growth, not death. To test this, MC3T3-E1 mouse osteoblasts were cultured *in vitro* with or without sodium nitroprusside (SNP), a widely used NO donor. We show that SNP [10^{-4} M] significantly induces thymidine incorporation by 13 fold. An NO scavenger, carboxy-PTIO ablated the SNP-induced thymidine uptake. To our surprise, SNP treatment did not affect osteoblast cell number suggesting that growth and death were occurring simultaneously. However, osteoblast necrosis and apoptosis could not account for the lack of a cell number change. Fractionation and Brd-U staining studies demonstrate that thymidine is incorporated predominantly into the nuclear fraction of osteoblasts. These findings lead us to a new hypothesis that [10^{-4} M] SNP cuases DNA damage, DNA repair, and consequently increased thymidine incorporation in osteoblasts.

dedicated to my family, for whose love and support has profoundly influenced my life and educational experiences

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

NO	**Nitric Oxide**
NOS	**Nitric Oxide Synthase**
SNP	**Sodium Nitroprusside**
LPS	**Lipopolysaccharide**
CGMP	**Guanosine 3'-5'cyclic monophosphate**
O_2	**Molecular oxygen**
CO	**Carbon monoxide**
CO_2	**Carbon dioxide**
IGF	**Insulin Growth Factor**
TGF	**Transforming Growth Factor**
FGF	**Fibroblast Growth Factor**
PTH	**Parathyroid Hormone**
TNFα	**Tumor Necrosis Factor alpha**
IL-1	**Interleukin-1**
INF	**Interferon-gamma**
BMP	**Bone Morphogenic Protein**
AP-1	**Activating Protein-1**
OPG	**Osteoprotegrin**
NF-KB	**Nuclear Factor Kappa Beta**
RANK	**Receptor Activator of NF-KB**
RANK-L	**Receptor Activator of NF-KB Ligand**
PGE_2	**Prostaglandin-2**
L-NAME	**N^G-nitro-L-arginine methyl ester**
L-NMMA	**L-N^G-monomethylarginine**
SNAP	**S-nitroso-N-acetyl-D-L-pencillamine**
FADH	**Flavine adenine dinucleotide hydrogen**
DNA	**Deoxyribonucleic Acid**
ATP	**Adenosine tri-phosphate**
DTTP	**deoxy-thymine monophosphate**
DUTP	**deoxy-uridine monophosphate**
HOGG1	**Human 8-oxoguanine glycosylase**
DNA-PKcs	**DNA-dependent protein kinase catalytic subunit**
LDH	**Lactate dehydrogenase**
Brd-U	**5'-bromo-2'deoxyuridine**

INTRODUCTION

This thesis provides a thorough background on the elucidation of nitric oxide (NO) as a cellular messenger in the mammalian system. We will get an appreciation for what bone is, and some of the factors involved in regulating its maintenance and remodeling. This thesis will also provide insight into why NO is important in regulating bone cell function, especially the effects of NO on bone-forming osteoblasts and resorbing osteoclasts. It has been shown that mechanical strain and sex hormones stimulate osteoblast production of low levels of nitric oxide that may be beneficial for bone remodeling and maintenance. On the other hand, cytokine-induced production of high levels of nitric oxide by osteoblasts and inflammatory cells has been shown to inhibit bone formation and possibly cause cell death. Based on these findings, the research conducted in this thesis was aimed at addressing two hypotheses. The first hypothesis is that NO regulates osteoblast cell growth. The second hypothesis is that NO causes osteoblast cell death.

LITERATURE REVIEW

1. Nitric oxide

Nitric oxide is one of the smallest molecules found in nature and was discovered over 200 years ago by Joseph Priestly.[1] Since then, biologists have studied inorganic nitrogen oxides in the biological nitrogen cycle and in food preservation, especially for curing meat. Nitrogen oxides are also important elements in air pollution. So environmentalists and chemists study their chemistry and toxicology as well.[1] The possibility that nitric oxide is a biological mediator in mammals was never questioned. However, it was not until the 1980's that investigators finally showed that NO is not only synthesized by mammalian cells but is important for regulating physiological processes and can be involved in pathological conditions.[2]

In 1981, Tannenbaum et al. reported that mammals synthesized NO by demonstrating that rodents excreted more NO_3^- than they ingested.[3] They also showed sterile irritants increased NO biosynthesis in animals. In 1985, Stuehr and Marletta reported that mammalian macrophages had the capacity to synthesize NO_2^- and NO_3^- if exposed to bacterial endotoxin (LPS).[4] This set the stage for Hibbs et al. who showed that L-arginine is a substrate for macrophage NO_3^- and NO_2^- biosynthesis and that N-guanidino substituted derivatives of L-arginine are potent competitive inhibitors of macrophage NO synthesis.[5] Soon after this observation, Palmer et al. reported that endothelial cells produce NO which causes vascular relaxation. They also demonstrated that L-arginine is the substrate for NO.[6] Thus NO is the biologically active intermediate

of the pathway. Furthermore in 1987, Ignarro et al. reported that NO is the endothelium-derived relaxing factor (EDRF) and linked NO production with cGMP increases in vascular smooth muscle.[7] Clinically, NO donors such as nitroglycerin and sodium nitroprusside, are widely used to treat high-blood pressure. Subsequently, NO has been identified as a major player in many systems including the neural system as a neurotransmitter and in the immune system as a cytotoxic effector molecule against foreign substances.[8]

1.1 Nitric oxide synthesis

NO is a highly labile free radical gas generated by nitric oxide synthase (NOS), which utilizes L-arginine and oxygen to form NO and L-citrulline.[9] There are three main forms of NOS enzymes, an endothelial (ecNOS), neuronal (nNOS), and inducible form (iNOS). The ecNOS and nNOS are regarded as the constitutive forms and are regulated by changes in free calcium [Ca^{++}_i]. Elevated [Ca^{++}_i] levels activate NOS by binding to calmodulin, which together binds to a calmodulin recognition site in NOS increasing its catalytic activity. The constitutive NOS enzymes are modulated by external stimuli that can influence [Ca^{++}_i] and ultimately cause cellular release of low (picomolar) amounts of nitric oxide.[10-12] In contrast, the inducible form (iNOS) is regulated at the transcriptional level and produces high (nanomolar) amounts of nitric oxide.[13-16] Pro-inflammatory cytokines are the major inducers of this enzyme. NO is also produced non-enzymatically in the stomach from nitrite[17] and from pharmacological donors such as sodium nitroprusside (SNP).[18]

1.2 Reactions of nitric oxide in vivo and in vitro

There are three main reactions accounting for the fate of nitric oxide in physiological solutions. NO binds to the ferrous heme iron of guanylate cyclase or other proteins, important for the activation of signaling transduction pathways via:

$$Heme\text{-}Fe^{2+} + {}^{\cdot}NO \rightarrow Heme\text{-}Fe^{2+}\,{}^{\cdot}NO$$

The major fate of NO is by its interaction with hemoglobin (Hb) in oxyhemoglobin or myohemoglobin to produce nitrate in vivo by a fast and irreversible reaction:

$$Hb\text{-}Fe^{2+}\text{-}O_2 + {}^{\cdot}NO \rightarrow Hb\text{-}Fe^{2+} + OONO^{-} \rightarrow Hb\text{-}Fe^{3+} + NO_3^{-}$$

Lastly, superoxide reacts irreversibly with NO to form the powerful oxidant peroxynitrite anion:[19]

$$^{\cdot}NO + {}^{\cdot}O\text{-}O: \rightarrow ONOO^{-}$$

Nitric oxide interaction with superoxide is known to cause lipid peroxidation and tyrosine nitration.[20,21] These reactions potentially explain why NO is such a short-lived molecule *in vivo* and spontaneously decomposes upon its interaction with oxygen and heme proteins.[22] *In vitro*, destruction of NO by reacting with superoxide in buffers is the most likely pathway for the short-lived NO. Upon release by its native synthesizing cell, NO diffuses to adjacent cells and acts locally. Given that NO is a small lipophilic molecule it

readily permeates cell membranes without using channels or receptors. NO actually has a diffusion coefficient higher than O_2, CO or CO_2, making it ideal for carrying a message into a cell.[23] The target cell receives the NO signal based on the concentration of NO present around the cell. This local concentration can be translated to a cellular signal by its unpaired electron binding to transition metals with high affinity. Aforementioned, its interaction with the heme-moiety of guanylate cyclase increases the synthesis of intercellular messenger cGMP. Its binding to ferrous groups is reversible and can be turned off after the gradient of NO has dissipated.[24] This is the main effector pathway of NO in vascular smooth muscle cells and platelets, but is evident in other cells and tissues as well. NO also reacts with sulphydryl residues and iron-sulfur centers in proteins. For example, it binds to the iron-sulfur center of aconitase found in the mitochondria, and alters its involvement in the Krebs Cycle.[25] It alters the ability of the mitochondria to carry-out cellular respiration by uncoupling the oxidative phosphorylation process. Furthermore, NO is known to impair DNA synthesis and cell division by reacting with tyrosyl radicals at the catalytic site of ribonucleotide reductase.[26] Lastly, nitric oxide can also cause nitrosylation[27] and under aerobic conditions NO is oxidized into nitrate (NO_3) and nitrite (NO_2).

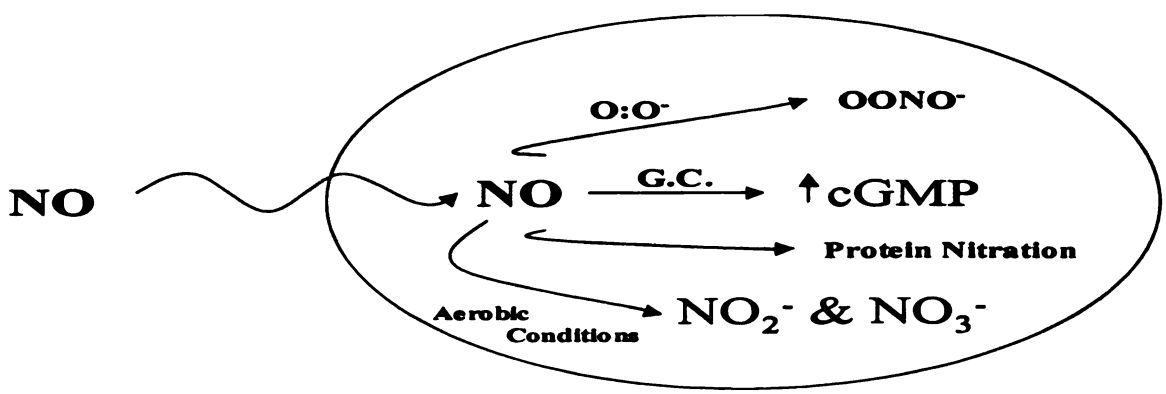

Figure 1 Main actions of NO in the cytosolic compartment.

2. Bone

Bone is a highly specialized form of connective tissue that provides structure, support, protection, and the ability to move about (Figure 2A). Mature bone can be seen as an inorganic mineral deposited on an organic matrix. This matrix is mineralized to provide rigidity and strength to the skeleton, while still maintaining some elasticity. The matrix and thus structure of bone is mostly comprised of a collagen fiber network, while the hardness of bone relies on the deposition of inorganic salts, such as calcium within the bone matrix.[28] Bone is the major source of calcium in the body and it regulates its concentration by controlling its release and deposition. For example, during hypocalcemic conditions bone releases calcium by initiating events that resorb bone to normalize plasma calcium levels. In contrast, when plasma levels are high, calcium can be deposited on the organic matrix produced by bone-forming cells (osteoblasts). So, bone is a repository for calcium in the post-absorptive state, and is the major source to tap into during fasting states. Overall, these processes are dependent on the coupling events that exist between osteoblasts and osteoclasts (bone-resorbing cells) and will be further addressed in section 2.3.

2.1 Bone tissues

Bone is comprised of two main types of bone tissue, compact and spongy (Figure 2B). Compact tissue is very hard and dense, and contains cylinders of calcified bone called osteons (Haversian canals)(Figure 3A). These cylinders are made up of lamellae, which are concentric layers of bone.[28] Lamellae contain lacunae, where the matrix

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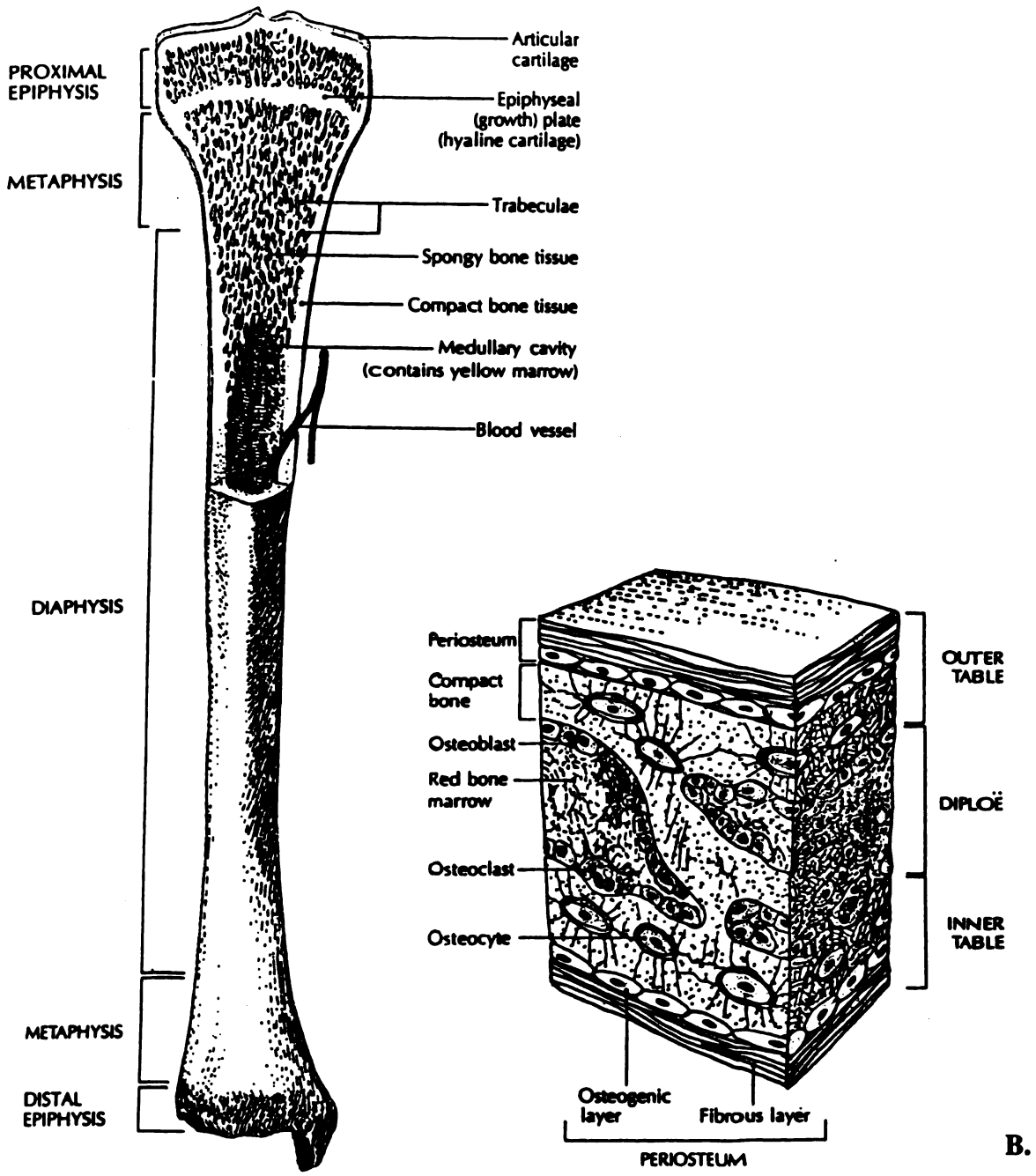

Figure 2 Gross Anatomy of a typical long bone. A. A typical long bone showing key anatomical features; while the interior is partially exposed. B. Illustration of compact bone surrounding spongy bone tissue and marrow. (Carola et al, *Human Anatomy and Physiology* 2nd Edition 1992)

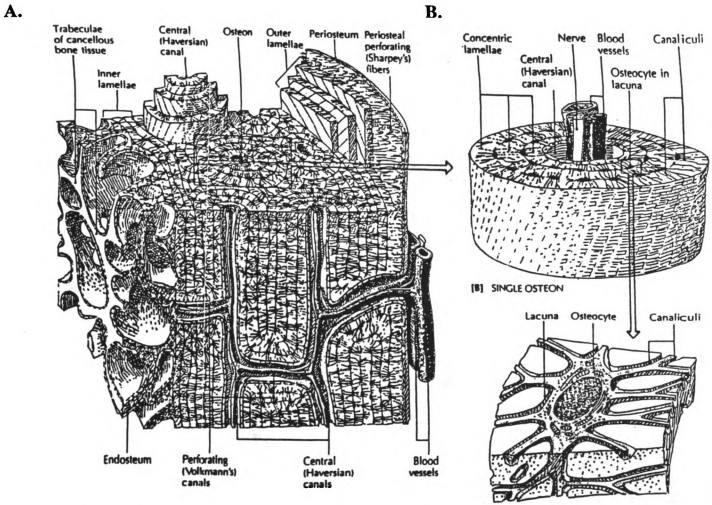

Figure 3 Compact tissue in bone. A. Compact bone tissue showing blood vessels, canals, and other internal Components. B. An enlargement of a single osteon. (Carola et. al., Human Anatomy and Physiology 2nd Edition 1992).

maintaining osteocytes reside.[28] Radiating from each lacunae are canaliculi that contain extensions of osteocytes, so that they can communicate with each other.[28] The central part of these canals contains the vasculature, nerves, and lymphatic supply for nutrient and waste export. The structure of the osteon provides the strength needed to withstand everyday mechanical strain or compressive force. The cancellous or spongy tissue is the other major tissue that comprises bone. Cancellous tissue has an interlaced pattern that sustains maximal stress and supports shifts in weight distribution.[28] Trabeculae are tiny spikes of bone tissue found in the interior structure of cancellous tissue.[28] These trabeculae are surrounded by calcified matrix or matrix hardened by deposition of inorganic salts.[28] Clinically, post-menopausal women exhibit a thinning of the trabecular compartment making them more susceptible to fracture.

2.2 Bone cells

Bone is comprised of many types of cells. Osteoblasts, osteocytes, and osteoclasts play key roles in the regulation of bone remodeling and therefore will be discussed in more detail (Figure 2B). A dynamic equilibrium must exist between bone formation and resorption to maintain skeletal homeostasis.[29] This equilibrium is dependent on mainly two types of cells, the bone-forming osteoblasts and the resorbing osteoclasts. These two cells are influenced by many factors in the bone microenvironment, which regulate their activity (IGF's, TGF's, PTH, etc. will be addressed in section 2.3). Osteoblasts are cells that produce an organic matrix that mineralizes upon deposition of inorganic salts such as calcium. They are derived from osteoprogenitor cells within the connective tissue of the mesenchyme. Osteoblasts are referred to as bone-forming cells because of their

capability to secrete collagen I and non-collagen proteins for the production of an organic matrix.[30] Upon maturation of this matrix, inorganic salts such as calcium are deposited on it to form mineralized bone. Osteocytes are the terminally differentiated osteoblasts that are located within the mineralized matrix and communicate directly with osteoblasts through their cellular processes. Osteocytes are also thought to assist with maintaining the matrix and furthermore may contribute to bone responses to mechanical force. Some reports speculate that osteocytes are involved in the continuous exchange of both the mineral and organic compounds between the blood and matrix.[31,32] Osteoclasts on the other hand, are the bone-resorption cells. Osteoclasts are large multi-nucleated cells derived from the monocyte/macrophage lineage that destroy the matrix produced by osteoblasts. Osteoclasts contain an abundance of lysosomal vesicles that secrete substances for resorbing bone, given the appropriate signal.[33] Osteoclasts attach to matrix by ligands such as integrin alpha vitronectin(v) B3 and av B5. This generally results in a sealed extracellular compartment, which is acidified through the action of H^+ ATPase located in the ruffled membrane of the osteoclast.[34] As noted above, resorption and formation are closely coordinated, and upon a chemical signal by resting osteocytes or osteoblasts, recruitment, activation, and differentiation of osteoclast precursors occurs. During bone remodeling, osteoclasts resorb an area of bone, before osteoblasts are recruited to that area to "fill" in the gap and form new bone.[35]

2.3 Growth factors involved in regulating bone remodeling and maintenance

There are many growth factors involved in regulating bone formation and resorption. Some factors such as cytokines, growth factors, and hormones stimulate bone formation by mediating stem cell progression to the osteoblast lineage. Fibroblast growth factor (FGF) and transforming growth factor-β1 (TGF-β1) are potent mitogens produced by osteoblast linage cells for periosteal osteoprogenitor and marrow stromal cells.[36] Bone-morphogenic proteins (BMP's) are important to cells responsible for inducing osteogenesis, in particular BMP 2, 4, and 7. BMP's are part of the large, the transforming growth factor-B (TGF-B) superfamily.[37-39] Their most striking characteristic in bone is to induce osteoblast-like phenotype in undifferentiated mesenchymal cells of embryonic or adult origin.[40,41]

The two most well studied factors that regulate bone turnover are vitamin D and parathyroid hormone (PTH). PTH is a peptide produced in the parathyroid glands and is secreted when blood calcium levels decrease. PTH's major function is to enhance osteoclast bone resorption to increase plasma calcium levels. One way that PTH stimulates bone resorption is by stimulating osteoblast secretion of interleukin-6 and macrophage colony stimulating factor (M-CSF), which activate neighboring osteoclasts.[42] Interestingly, PTH is also known to cause an increase in osteoblast number and collagen synthesis in culture, and when given intermittently, increases bone formation. Vitamin D is also a major player in Ca^{++} absorption from the intestine and bone. In the small intestine, vitamin D induces expression of calcium-binding protein (calbindin) which facilitates Ca^{++} absorption. This increases plasma calcium and can increase bone

formation. Vitamin D is furthermore directly involved in bone formation and monitoring mineralization of osteoid. In vitamin D deficiency, osteoid accumulates without mineralization and rickets develops. Vitamin D also induces Ca^{++} resorption form bone by stimulating osteoclast activity through osteoblasts. In bone specifically, vitamin D increases the recruitment, differentiation, and fusion of hemapoietic precursors into active osteoclasts, which carry out the resorptive activities.

Sex steroids are also essential for maintanence of normal bone volume. Estrogen is an important steroid hormone that influences both osteoblast and osteoclast function. Estrogen receptors have been found in human,[43] mouse[44] and rat[45] osteoblasts and has been shown to induce osteoblast cell proliferation.[44,46,47] Estrogen also plays a role in extracellular matrix maturation by inducing alkaline phosphatase expression in osteoblasts.[47-50] Furthermore, estrogen influences 1,25 dihydroxyvitamin D-receptor levels[51] and PTH stimulation of cAMP levels.[42] While estrogen affects osteoblast function, it also regulates osteoclast function directly or indirectly through cytokine or growth factor production by osteoblasts.[52,53] Estrogen suppression of IL-6 is thought to be important in suppressing bone resorption. In fact, the knockout of IL-6 gene expression in mice prevented bone loss after oophorectomy. Moreover, estrogen directly acting with estrogen receptors on osteoclasts, may inhibit bone resorption.[54] There have been many conflicting reports, mainly due to the cell system used, about how estrogen regulates osteoblast and osteoclast function, but it seems there may be a direct and indirect means in which estrogen affects their function. Altogether, osteoblast mediated effects of estrogen on osteoclasts are dependent on cytokines such as M-CSF, TNFα, IL-1 and IL-

6,[55] which stimulate osteoclast resorption by increased proliferation and differentiation of osteoclast precursors.[34] Other steroids that play a role in bone, but will not be addressed are testosterone, progestins, and androgens.

Recently, osteoblasts were shown to produce a surface-residing molecule that is essential for osteoclastogenesis, called osteoprotegrin[56] (Figure 4). OPG is a receptor that

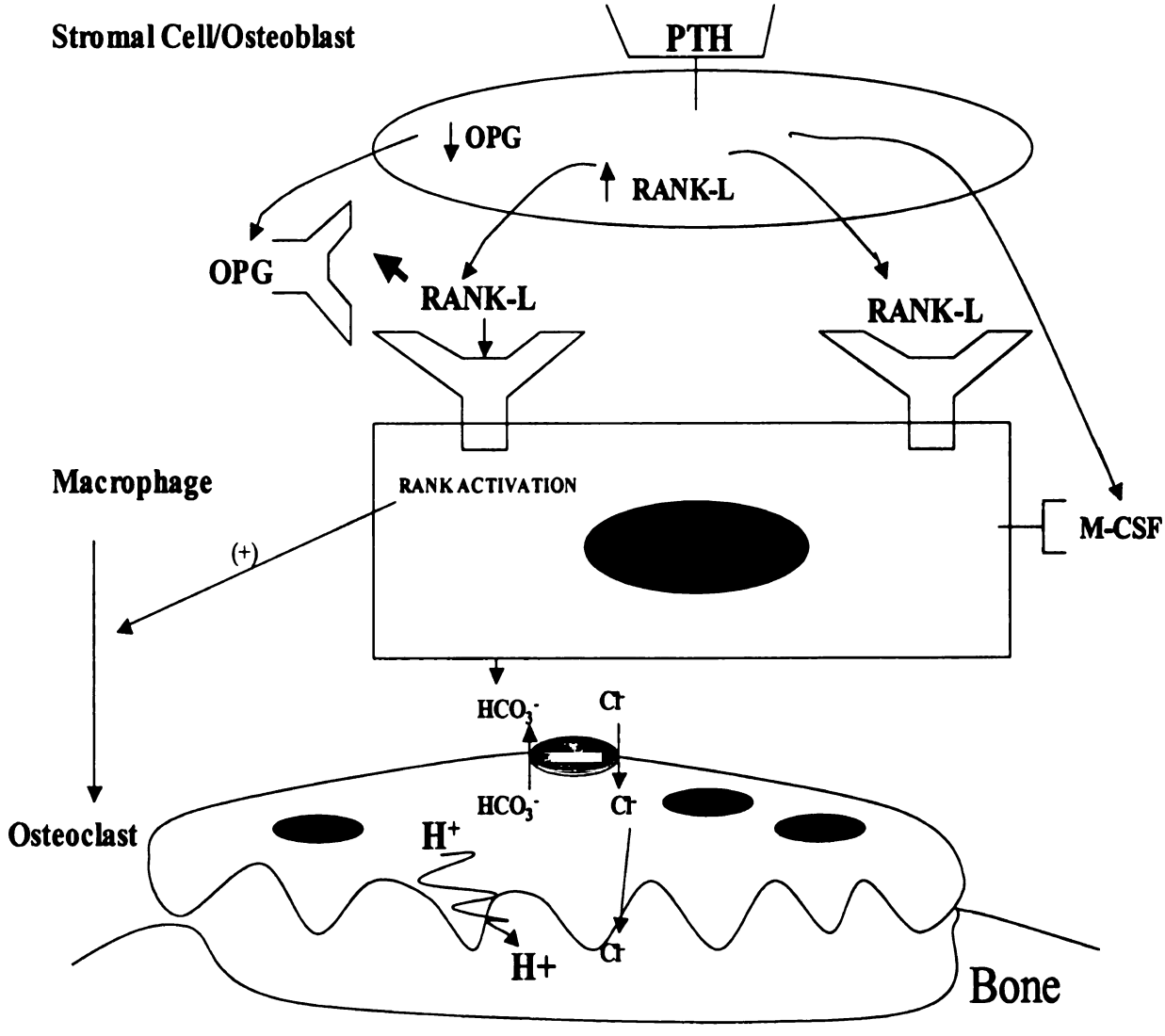

Figure 4 Mechanisms of osteoclastogenesis and osteoclastic bone resorption.[58]

blocks osteoclastogenesis in mice by binding RANK-L. Mice deficient in OPG have accelerated osteoclastogenesis and develop osteoporosis.[56] RANK (Receptor activation of NF-KB) is the receptor on macrophages that binds RANK-L to stimulate osteoclastogenesis. Thus, OPG is a "decoy" receptor, which competes with RANK for RANK-L.[57] RANK-L is up-regulated by PTH (Figure 4), vitamin D, IL-11 and PGE_2, which are stimulators of bone resorption as well. Osteoblasts and osteoclasts are in close apposition and it seems osteoblasts are directly involved in regulating bone resorption through the aforementioned factors.

When the dynamic equilibrium between bone formation and bone resorption is altered, bone diseases arise. Excessive bone formation is one of the hallmark signs of altered bone formation and resorption.[59] Osteopetrosis, a bone disease marked by excessive amounts of bone, results from reduced bone resorption relative to bone formation. Elster et al. (1992) reported that the inadequate osteoclast resorption causes a thickening of the cortical region and a decrease in the size of the medullary space as well as sclerosis in the base of the skull.[60] Some of the aforementioned factors controlling the coupling events between osteoblasts and osteoclasts are currently under investigation to determine the exact cause of this disease. The other major form of bone disease is marked by reduced bone mass, which when greater than 2.5 standard deviations of the average poplulation bone mineral density is called osteoporosis. Typically, osteoporosis results from increased bone resorption relative to bone formation.[61] Osteoporosis is primarily evident in post-menopausal women and estrogen deficiency. For example, it has been shown that estrogen increases skeletal resistance to PTH-induced resorption,[62]

which may partially explain the increased bone resorption evident in osteoporosis. Estrogen stimulates these factors, and their abundance would decrease in estrogen deficient situations.[63,64] Many treatments today are directed toward estrogen replacement therapy in attempt to prevent further bone loss from occurring and to possibly stimulate new bone formation.

3. Nitric oxide and its beneficial effects on bone

Three major forms of nitric oxide synthase exist, however it is the constitutive endothelial form (ecNOS) and the inducible form (iNOS) that are found in osteoblasts.[65-68] NO is thought to have beneficial effects on bone and it has been shown that osteoblasts produce NO in response to anabolic stimuli such as sex steroids,[69,70] mechanical loading,[71-73] fluid flow,[74] and also during fracture healing.[75] For example, Ralston and Armour reported first that estrogen increased NO production by human osteoblasts through the activity of the cNOS Ca^{++} dependent isoforms.[76] In conditions where estrogen is deficient (osteoporosis), Wimalawansa et al. reported that administration of the NO donor nitroglycerin prevented ovariectomy-induced bone loss.[77] In rats given β-estradiol, administration of the NOS-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) abolished the protective effect of 17-β estradiol suggesting that NO plays a critical role in estrogen effects on bone.[77] Altogether, these reports support the role of NO in regulating bone cell function.

It has also been reported that mechanical loading is beneficial for bone and previous studies have shown that NO is released by osteoblasts in response to mechanical loading.[71-73] Specifically, transient rapid increases in NO release were stimulated by strain in both rat long bone-derived osteoblast-like cells (LOBs) and embryonic chick osteocytes (LOCYs) in monolayer culture. Examination of NOS expression in the skeleton demonstrates that load-bearing rat long bone periosteal osteoblasts and cortical bone osteocytes expressed the endothelial form of NOS (ecNOS), whereas in non-load bearing calvariae there where no detectable levels of ecNOS in osteocytes and little in

osteoblasts. Altogether, load bearing bones release NO in response to loading *in vitro*, presumably through ecNOS expression.[73] Mechanical loading is known to induce bone formation, which stimulates rapid and continuous release of NO by osteoblasts as a result of ecNOS activity.[74] Fluid-flow occurs in bone by plasma leaking from venous sinusoids in the bone marrow space and is driven radially outward by a relatively steady transmural pressure gradient that exists between the vascular and lymphatic systems at the periosteal surface.[74] Thus, fluid flows through the canaliculi and over osteocytes. Mechanical loading, such as running, jumping, or walking, creates an increased fluid-flow in bone that stimulates bone formation at sites of mechanical strain. This fluid-flow creates a shear stress on osteoblasts leading to induced NO production. Shear stress is the force felt by osteoblasts in response to fluid-flow across them. It has been suggested that nitric oxide's involvement in this process is beneficial for maintenance of healthy bone. In contrast, decreased fluid-flow as seen in immobile states such as reduced physical activity or prolonged bedridden hospital stays could contribute to the bone loss associated with these conditions. These reports support clinician's medical advice to stay active as we age, so that we maintain healthy bone, which is constantly remodeling and repairing itself in part to suit the needs of the skeleton to maintain structural integrity at sites of mechanical strain.

NO also plays a role in fracture healing as demonstrated by Diwan et al using a rat femur fracture-healing model.[75] While NOS levels were undetectable in controls, following fracture, iNOS mRNA expression, protein, and enzymatic activity increased in rat femoral fracture callus, with maximum activity at day 15. Constitutive isoforms of

NOS (ecNOS and nNOS) were induced slightly later and were gradually increased throughout day 30. Suppressing the NO effect on fracture repair by an NOS inhibitor (L-NAME), reduced the cross-sectional area of bone by 15% and failure load by 45% on day 24 following fracture. Furthermore, delivering NO to the fractured site using carboxybutyl chitosan NONOate increased cross-sectional area by 30 % compared to the inhibitor group. This report also supports the role of NO in bone remodeling and repair.

4. Nitric oxide and its negative effects on bone

Nitric oxide can have negative effects on bone as well. NO is capable of being synthesized by osteoblasts in response to catabolic factors such as cytokines (IL-1, TNFα, and IFN-γ), endotoxins, and LPS, which are prevalent in inflammatory conditions such as rheumatoid arthritis, osteoarthritis, and periodontal diseases.[78-80] Cells generally do not produce biologically significant amounts of NO upon single treatment of cytokines (IL-1, TNFα, and IFN-γ), but upon combination of cytokines they work synergistically to generate higher NO concentrations. These findings have been reported in macrophages,[81] neutrophils,[82] mesangial cells,[83] hepatocytes,[84] chondrocytes,[85] in mouse osteoblasts,[78,86] human osteoblasts[87] and in osteosarcoma cells.[88] Reports have shown that NO at high concentrations inhibits osteoclast activity and differentiation.[89-91] While others demonstrate enhanced osteoclast activity and bone resorption at low NO concentrations.[62,63,80] Differences between these findings suggest that NO effects are dose-dependent. Most previous works have focused on how NO effects osteoclast function, whereas few studies have examined its effects in osteoblasts. Being that there is a dynamic coupling event between osteoblasts and osteoclasts, understanding the role of NO effects on osteoblast may provide insight how NO plays a role in this coupling event. High concentrations of NO have been shown to prevent osteoblast growth in response to cytokine stimulation as well.[87,92] This abrogation of synthesis was partially restored by using L-NMMA, a NOS inhibitor.

4.1. Inflammation and disease

The inflammatory response is a sequence of events that follows tissue damage caused by a wound or by invasion of a pathologic microorganism. The classic features of inflammation are redness, swelling, heat and pain. The complex series of events that occurs in the inflammatory response involves a plethora of chemical molecules derived from many sources, including invading microorganisms, cells damaged by the response to tissue damage, and some constituents of the blood and lymph systems.[93] What mediators of inflammation are of importance to the work in this thesis are pro-inflammatory cytokines, such as TNFα, IFN-γ, and IL-1. These cytokines may be involved in the bone loss seen in rheumatoid arthritis and will be discussed shortly. Specifically, TNFα and IL-1 have been shown to be mediators of inflammatory bone loss,[94] whereas IFN-γ has been reported to selectively inhibit cytokine-induced bone resorption.[95] Furthermore, nitric oxide concentrations have been shown to be elevated in inflammatory conditions, and are of particular interest for the work in this thesis. It has also been suggested that nitric oxide may play a role in the increased blood flow to the infected area by its vasodilatory effects. However, unregulated NO production may become destructive to an organism as is evident in many disorders such as autoimmune disease, immune rejection of allografted organs, and sepsis.

Rheumatoid arthritis is an inflammatory condition in bone also characterized by rapid bone loss. The exact cause of this bone loss in unknown, but it has previously been reported that nitric oxide (NO) may be involved in this process. It has been demonstrated that suppressing arthritis was possible by inhibiting nitric oxide synthase

activity, the enzyme involved in nitric oxide production.[96] Grabowski et al. also reported that cells derived from the human joint were able to generate NO.[97] In particular, synovial fibroblasts, articular chondrocytes, and osteoblasts were cultured from tissues of patients undergoing hip replacement surgery. Little production of NO was generated after culture, but all cells produced large amounts of NO following a cytokine mixture of IL-1, TNFα, and IFN-γ. This suggests that these cells may be involved in the inflammatory response especially in joints where high concentrations of cytokine-induced NO have been reported. This area of research is now intensively studied and is one reason or conducting the work in this thesis. Altogether, these findings suggest a dose-dependent effect of NO in bone. In this thesis, we will examine the direct effects of NO generated pharmacologically by SNP on mouse osteoblasts in attempt to delineate its role in bone.

5. Cell death by apoptosis and necrosis

There are two major forms of cell death: necrosis and apoptosis. Cell death is an irreversible process that once initiated, cannot be prevented from occurring. Apoptosis is referred to as "programmed cell death", in which the cell executes its internal program to shut down.[98] Many molecular events occur within the apoptotic cell. Early apoptosis features cell dehydration. This leads to condensation of the cytoplasm, followed by changes in the cytoskeletal framework and cell morphology. The predominant characteristic is condensation of nuclear chromatin. This condensation starts out at the periphery of the nucleus, then the nuclear envelope disintegrates, lamin proteins undergo proteolytic degradation, and finally the nuclear DNA becomes fragmented. This highly condensed DNA makes it easy to detect apoptotic cells by using fluorescent dyes, which stain intensely in damaged DNA. The fragmented DNA is then packaged into segments of the plasma membrane and referred to as "apoptotic bodies". In contrast, necrosis is referred to as "accidental cell death or cell murder." It is characterized as cell swelling followed by rupturing of the plasma membrane.[98] This process is generally cytotoxic to surrounding cells, due to the release of proteolytic enzymes.[99] Nuclear chromatin shows patchy areas of condensation, less so than apoptotic cells, and the nucleus undergoes slow dissolution.[98]

5.1 Nitric oxide and cell death in bone

NO has been demonstrated to be an important mediator of apoptosis. There are many reports that NO ($>10^{-4}$ M) damages cells and causes cell death via apoptosis. This has been elegantly shown in the studies of Mogi et al. who demonstrated that cytokine

(IL-1, TNFα, & INF-γ) induced NO production causes a potent decrease in MC3T3-E1 osteoblast cell proliferation and eventually induces apoptosis.[100] They also reported that exogenous NO production via S-nitroso-N-acetyl-D-L-pencillamine [$5x10^{-4}$ M] caused DNA fragmentation greater than that seen using the cytokine mixture. Furthermore, Damoulis and Hauschka reported that concentrations (>10^{-4} M) of NO induced apoptosis in MC3T3-E1 mouse osteoblasts.[92] They also showed that various combinations of cytokines reduced cell viability as measured by MTT assay and trypan blue exclusion.

5.2 DNA damage and repair mechanisms

DNA is the genetic material housed within the nuclei and mitochondria of cells that carries that "master blueprint" to an organism. Although DNA is dynamic in structure, it is readily susceptible to changes, both "macro" and "micro" in nature. "Macro" changes are characterized as the translocation of large nucleotide sequences, which alters the gene expression in living cells.[101] Also DNA is subject to "micro" changes such as single nucleotide replacement.[102-107] Many of these changes are a consequence of errors during DNA replication, recombination, and repair itself. Other base changes arise due to chemical and spontaneous induced modifications to chemical bonds that comprise nucleotides under normal physiological conditions These changes can bring about damage to all components of DNA (sugars, bases, and phosphodiester linkages). Modifications that cause DNA damage elicit DNA repair processes to correct the altered DNA.

DNA repair is a major defense against environmental or chemical-induced modifications of DNA. It is apparent in all organisms including bacteria, yeast, drosophila, fish, amphibians, rodents and humans. This defense system ultimately minimizes lesions leading to severe mutations, replication errors, and persistent DNA damage and genomic instability.[108] There are many forms of DNA repair, some include: direct reversal, nucleotide excision, base excision, and recombination.

Direct reversal repair is elicted when, for example, ultraviolet light forms pyrimidine thymine dimers.[109] Enzymes called DNA photolyases, bind directly to the pyrimidine dimer, and transfer an electron to the dimer, to split it into monomers. Pyrimidine dimers generated by other means are repaired by nuclear excision repair as well. Repair occurs by multi-subunit enzymes (UvrABC endonucleases) via an ATP-dependent reaction, which excises the oligonucleotide containing the lesion. The excision is then replaced by a DNA polymerase, which utilizes the undamaged template strand as a guide for the incorporation of the correct base, followed by ligation by DNA ligase. UvrABC endonuclease also recognizes displacements of bases from their normal positions (bulky-adducts) and is activated by a helix distortion.[109]

Base excision repair is another form of excision DNA repair. Base excision occurs under normal physiologic conditions when DNA contains a damaged base, caused by spontaneous deamination of adenine or cytosine to yield hypoxanthine and uracil residues, respectively.[116] These mutations are excised by DNA glycosylases that cleave the glycosidic bond, leaving a deoxyribose residue in the backbone. Such apurinic or

apyrimidinic (AP) sites are also generated at glycosidic bonds by spontaneous hydrolysis. The altered residue is cleaved on one side by an AP endonuclease and removed by actions of DNA polymerase and some exonucleases. The gap is filled in by DNA polymerase I and ligated by DNA ligase. An interest of the research in this thesis is that deamination of cytosine to uracil (RNA base) in DNA can be mutagenic and is quickly replaced by thymine through excision repair. This repair pathway will be a concern of the research found in this thesis and will be further addressed in the discussion section.

Furthermore, dealkylation of alkylated nucleotides by alkyltransferases, is highly mutagenic because it causes the incorporation of thymidine instead of cytosine into DNA, yielding D^6-alkylguanine residues among other products.[109] These lesions are mismatches that require base-excision repair. They are repaired by methylguanine-DNA methyl-transferases, which transfer offending alkyl groups directly to its own cysteine residues. Mismatch repair generally occurs following DNA replication as a "spellcheck" process. A series of proteins actually scan the DNA and look for incorrectly paired bases (unpaired bases), which distort the double helix.[110] The incorrect base is removed as a short-stretch and DNA polymerase gets a second try to get DNA replication correct.

The last method of repair that will be addressed is recombination or post-replication repair. Sometimes damaged DNA undergoes replication before it has the opportunity to repair a lesion caused by the aforementioned processes.[109] This process exchanges corresponding segments of sister DNA strands, thereby placing the injured

DNA segment next to the undamaged strand where the gap is in position to be corrected and sealed by DNA ligase.[109]

5.3 Nitric oxide, DNA damage & repair

Nitric oxide as mentioned above can cause DNA damage, but the exact mechanism by which this occurs is still unknown. In response to DNA damage, the cell undergoes cell arrest to repair the DNA before replicating it.[111] This mechanism prevents mutated DNA from being duplicated. If the repair system is inhibited or down regulated, an accumulation of DNA lesions can occur resulting in severely mutated DNA. These cells either duplicate mutated DNA or undergo cell death. Many reports have shown that NO interacts with or inhibits the activity of enzymes involved in DNA repair. In particular, it has been reported that NO directly inhibits hOgg1, a key base excision repair enzyme, responsible for base excision repair of 8-oxoguanine.[112] High levels of NO can also attenuate cell-cycle progression at the G1 phase.[111] Expression of p53, the tumor suppressor gene, and p21 (WAF1/CIP1), a cyclin-dependent kinase inhibitor, is induced during this process. Apoptosis is the consequence of DNA damage and subsequent expression of p53 and p21. Conversely, nitric oxide has been shown to induce the expression of DNA repair enzymes such as the DNA-dependent protein-kinase catalytic subunit (DNA-PKcs), one of the enzymes involved in repairing double stranded DNA breaks.[113] This mechanism is thought to protect the cell population. Taken together, it seems the role of NO in regulating DNA repair and damage is concentration dependent.

6. Nitric oxide donors

To study the effects of NO in cell culture, nitric oxide donors can be used to generate NO. When using NO donors, one must recognize that NO released is quickly inactivated by reacting with O_2, O_2^-, thiols, heme iron, and by participation in nitrosation reactions.[114] Along with these reactions, two other parameters modify the effects of donors: the redox form of NO and the rate of release. Three different redox forms exist: $NO^\cdot$, NO^-, and NO^+.[115] The form generated from NO donors depends on many factors, including the pH, presence of redox substances in the culture medium, and the donor make-up itself.[116] The rate of release of NO is also dependent on pH, temperature, and donor composition. A caveat of using NO donors is that they have widespread and different effects, even though the same donor concentrations are used. In addition, effects seen can result from NO directly or from a donor compound byproduct. To address this issue, NO scavengers are now used to distinguish between NO effects and non-physiologic effects induced by donor byproducts.

6.1 Measurements of nitric oxide

Since the half-life of NO is on the order of seconds, measurement is quite difficult although chemiluminescent techniques can be used.[117] More often though, NO is measured indirectly by measuring the conversion of radiolabelled L-arginine to L-citrulline[118] or by measuring the accumulation of NO_2^- and NO_3^- in biological fluids such as plasma, urine, and synovial fluid.[3] *In vitro*, nitric oxide levels are generally determined by measuring a stable end product, nitrite (NO_2^-) in the culture media. The measurement

is through the Griess Reaction.[119] It has previously been shown that nitrite production is directly proportional to NO derived from L-arginine.[95,102]

7. Approaches to address hypotheses and findings

Taken together, the findings in the introduction demonstrate that nitric oxide can modulate osteoblast phenotype, but the effects could be critically dependent on the concentration and rate of NO released. Therefore, given the potential bone anabolic effects of nitric oxide, we subjected a non-transformed MC3T3-E1 mouse osteoblast cell line to sodium nitroprusside (SNP, Figure 4), an NO donor, in attempt to gain a better understanding of NO effects on osteoblast growth and function. SNP is a widely used NO donor today, clinically and in research studies. SNP spontaneously releases NO in a non-linear manner, but the mechanism of release remains obscure.[117] The concentrations we used released anabolic levels of NO. We hypothesize that SNP will increase osteoblast growth. We tested this hypothesis by examining osteoblast growth via thymidine incorporation, which measures the amount of radiolabeled tritiated thymidine uptake into cells and is usually indicative of growth. Although thymidine uptake was increased more than 13 fold by the addition of SNP, we did not see a change in cell number or DNA levels. This observation led us to our second hypothesis that NO also causes cell death. Cell viability was determined by microscopic cell counts using trypan blue exclusion and lactate dehydrogenase (LDH) measurements. Cell death was further examined by propidium iodide staining of nuclear fragmented DNA. Propidium staining is a viable method of examining apoptotic cells. The nuclear and cytosolic compartments were analyzed for thymidine incorporation to determine exactly where the thymidine uptake was being incorporated and 5'-bromo 2'-deoxyuridine (Brd-U) staining was used to verify thymidine localization. We report that SNP [10^{-4} M] significantly induces thymidine incorporation in MC3T3-E1 osteoblasts. No change in cell growth or cell

death was evident between SNP treated and untreated osteoblasts. *SNP-induced* thymidine incorporation was predominantly found in the nuclear fraction versus cytosolic (mitochondrial containing) fraction of cells. This was verified by Brd-U incorporation, which demonstrated that the percentage of incorporation was similar between SNP treated and untreated conditions. However, there was more intense staining in nuclei of SNP treated cells. Taken together, our results show that [10^{-4} M] SNP doesn't stimulate osteoblast growth or death, but suggests that SNP causes DNA damage and consequently DNA repair in osteoblasts.

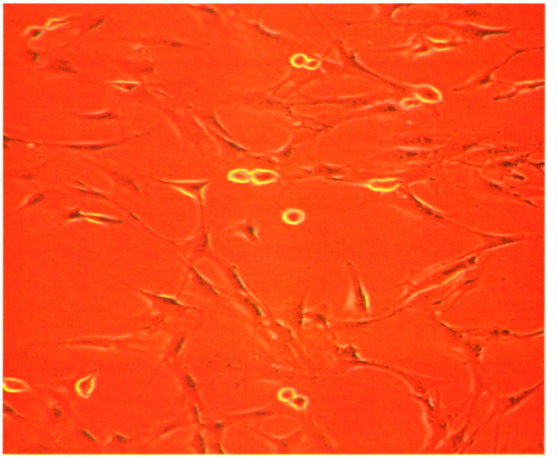

Figure 5 Illustration of MC3T3-E1 mouse osteoblasts. This is a microscopic image of a sub-confluent culture of day 3 osteoblasts (As described in Methods).

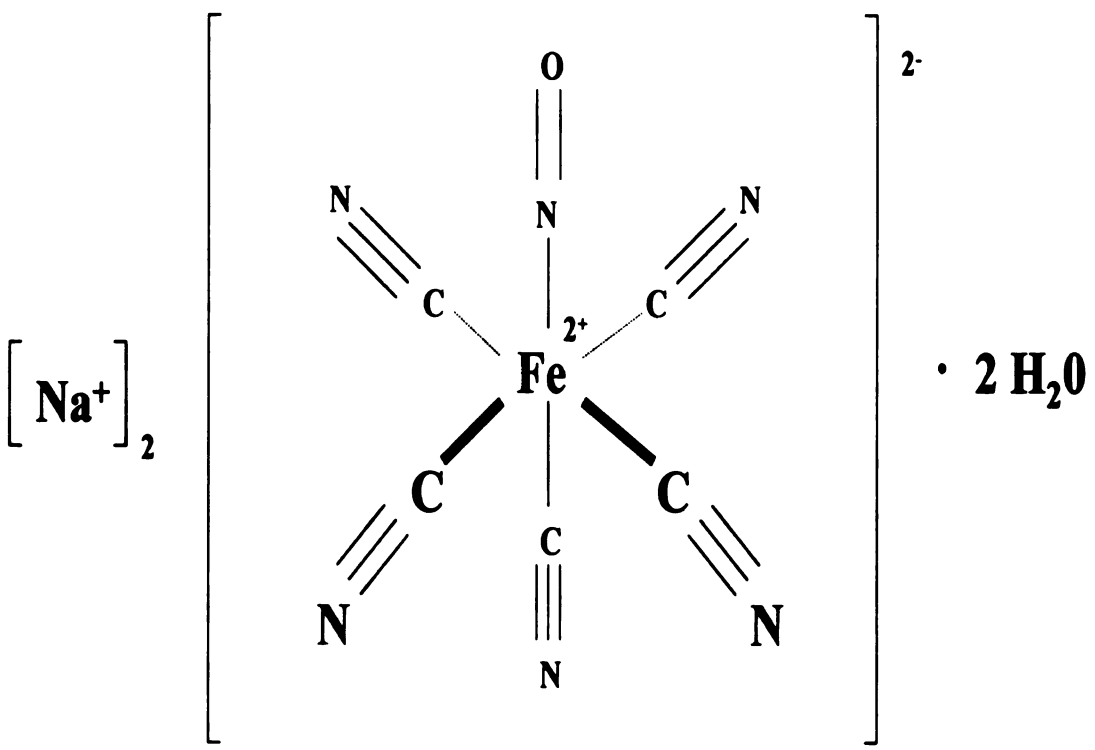

Figure 6 Chemical structure of sodium nitroprusside (SNP).

MATERIALS AND METHODS

Cell culture system:

MC3T3-E1 mouse osteoblast were plated at $4x10^4$ cells per well, in 6-well tissue culture plates.[120] The cells were fed 24 hours later with alpha-MEM (Gibco) supplemented with 10% fetal calf serum. The next day, osteoblasts were treated with sodium nitroprusside (SNP, Sigma) and/or carboxy-PTIO (Cayman Chemical) and in some cases refed (however, we did not find differences in responsiveness between unfed versus fed cultures). SNP concentrations ranged from 10^{-6} to 10^{-4} M, and carboxy-PTIO concentrations were between 10^{-4} and 10^{-3} M.

Thymidine Incorporation:

Twenty-four hours after treatment with nitric oxide donors or scavengers of NO (carboxy-PTIO) MC3T3-E1 cells were incubated for 2 hours with 4uCi/ml media tritiated thymidine at 37 degrees Celsius in an environmental incubator. Following incubation, the medium was aspirated and the monolayer was rinsed twice with cold PBS. DNA was precipitated by incubating cell layers in 10% TCA on ice for 5 minutes, repeating this step, and then solubilizing the precipitate in 10% SDS. Each well was scraped and its contents were added to a scintillation vial with scintillation fluid for counting. Thymidine incorporation (cpm) was normalized per ug of DNA. Thymidine incorporation into nuclear versus cytosolic (mitochondrial containing) fractions was done by pulse labeling osteoblasts and then isolating the cell fractions. Nuclear extracts were obtained by scraping MC3T3-E1 cells in PBS, washing three times with PBS, pelleting, and then

resuspending in NP40 lysis buffer[121] (H_2O, 1M Tris pH 8, 2-beta mecaptoethanol, and NP40). Samples were iced for 10 minutes and then nuclei were pelleted at 3000g for 5 minutes. The entire volume of supernatant represented the cytosolic fraction while nuclei were further washed with NP40 lysis buffer. Isolation of a predominantly nuclear fraction was verified by trypan blue staining. DNA was precipitated with 10% TCA and processed for measurement of thymidine incorporation as outlined above. For 8 and 48-hour thymidine incorporation, osteoblasts were pulsed with 4uCi tritiated thymidine 8 and 48 hours after SNP treatment respectively.

DNA Measurements:

MC3T3 cells were washed twice with PBS and harvested for DNA measurement as previously described.[120] Specifically, cells were lysed with 10mM EDTA and stored at 4 degree Celsius. One to two days later, 1M KH_2PO_4 was added to neutralize samples (25ul), which were then sonicated at 3 power, 80% cycle for 10 seconds (Artek sonic dismembrator model 150, Artek Systems Corporation). Hoechst dye (33342, Sigma) was added to achieve a final concentration of 1.2ug/ml and DNA levels measured by fluorometry. Absorbance values were normalized to a DNA standard for final DNA content.

Nitrite Measurements:

Nitrite was measured using the Griess Reaction colorimetric assay.[119] At the time of cell harvesting, 100ul of culture media was put into a 96-well tissue culture plate. Then, according to the manufacturer's protocol (Cayman Chemical), 50ul of Griess

Reagent R1 was added followed by an addition of 50ul of Griess Reagent G2. The plate was mixed and allowed to sit at room temperature for ten minutes, before reading at 540nm absorbance on an ELISA colorimetric reader. Background absorbance was read at 690nm (well no media or cells). The final nitrite absorbance was determined by subtracting the 690nm absorbance from the 540nm absorbance. A nitrite standard curve was used to provide final nitrite concentrations (uM).

Microscopic counting and trypan blue exclusion:

Twenty-four hours after NO treatment, cells were trypsinized and counted in a hemocytometer. Trypan Blue at a concentration of 0.4% (4mg/ml) was added to the cell mix to determine the percentage of viable cells excluding trypan blue stain. Triplicate samples were counted per condition and more than 3 separate experiments were carried out.

Propidium Iodide Staining:

MC3T3-E1 osteoblasts were plated at $1x10^4$ cells in 12-well plates. At the time of harvest (24 hours after treatment, day 3 osteoblasts), cells were fixed in a final concentration of 70% ethanol (added directly to media) and stored at 4 degree Celsius. Before removing the ethanol, plates were spun at 1400 rpm to bring osteoblasts floating in the media to the bottom of the plate. The PI staining solution was 200ug PI/ml PBS. DNAase free RNAase was added to the final PI solution before addition into cell culture and incubation at 37 degree Celsius for 20 minutes. Cells were then examined by fluorescent microscopy. Cell analysis was done by counting 12 random fields per well of

a 12-well culture plate at 20X magnification on a fluorescent microscope. Fragmented nuclei were compared to osteoblasts nuclei following a 24 hour treatment with 20ng/ml TNFα to induce apoptosis.[92] Data represents three separate experiments. Cell counts per experiment ranged between 1200-2400 per condition.

LDH Meausurements:

LDH measurements were conducted according to the manufacturer's protocol. Cells were treated with SNP [10^{-4} M] 48-hours after plating as explained above. On day 3, a 1 ml sample of media was taken. To 100ul of reaction mixture (Cytotoxicity detection kit, ROCHE), 100ul of sample media was added and incubated at 37 degrees Celsius for 30 minutes. Absorbances were measured at 490nm using an ELISA reader. Reference wavelength was measured at 680 nm. Reference wavelength was subtracted from sample absorbance to give the final LDH measurement (Arbitrary Units).

5'-Bromo- 2' deoxyuridine (Brd-U) Analysis:

Osteoblast cells were plated on cover slips at a density of $4x10^4$ in 6-well plates. On day 2, cells were treated with SNP [10^{-4} M]. On day 3, Brd-U 10uM(Becton Dickenson) was added to the cells for 2 hours before fixing in 70% ethanol. Cells were washed three times in PBS, before adding a 4N HCL/PBS solution to denature the DNA. After twenty minutes, the cells were neutralized in PBS pH7. FITC-conjugated Anti-Brd-U was added with PBS containing 1%BSA and 0.5% Tween 20, and placed in a CO_2 incubator at 37 Celsius for 45 minutes. The cells were then examined by fluorescence microscopy. Cells were viewed at 20X and 40X magnification on a reflected light

fluorescent microscope (Olympus BH2-RFL Japan 203018 model). The intensity of Brd-U nuclear labeling was derived using Image Pro Plus 4.1 Software, specifically the line profile measurement function. The number of cells measured for Brd-U intensity were control (471) and SNP-treated (603). Four subgroups were determined relative to nuclear foci. Group I is highly fluorescent with no nuclear foci. Group II is weakly fluorescent with no nuclear foci. Group III displays few (<15) unequally distributed foci. Group IV displays more (>15) nuclear foci than Group III.

Thymidine kinase assay:

MC3T3-E1 osteoblast (SNP treated for 24 hours and control) were washed in cold PBS twice before scraping into an microcentrifuge tube. Cells were spun at 600g and pelleted. Supernatant was removed before adding 100ul of cold NP40 lysis buffer. The pellet was resuspended and iced for 3 minutes before centrifuging in 4 degree Celsius for 30 minutes. The supernatant was stored in –80 degree Celsius until analysis. Upon analysis, 15 ul of extract was mixed with 12.5ul of 5X buffer (250 mM Tris pH 8, 75 mM NaF, 18 mM 2-beta mercaptoethanol, 12.5 mM $MgCl_2$, 0.4 mM thymidine, H_2O and +/- 25 mM ATP), 250 uCi/ml ^{3}H-thymidine, and 22.5 ul H_2O. This was set up on ice, then incubated at 37 degree Celcius for 30 minutes. The reaction was stopped by boiling for 5 minutes, then spotted in triplicate on DE81 paper (10ul/spot). Filters were prepared by washing 2X with 1 mM ammonium formate, then washed 1X with methanol. The washes were done in beakers and swirled on a rotator for 10 minutes. Filters were dried and put in scintillation vials then ^{3}H-TMP was eluted with 1 ml of 0.1 M HCl/0.2 M KCl

and shaken on a rotator for 15 minutes. Scintillation fluor was added and contents shaken until the solution cleared before being read with a scintillation counter.

FACS analysis:

Following a 24 hours treatment with SNP [10^{-4} M] MC3T3-E1 cells (treated and untreated) were trypsinized, spun down, and resuspended in 1 ml of PBS. Cells were washed twice and 1 ml PBS + 10% serum was added. Then 10 ml of cold ethanol was added and the samples stored at –20 degree Celsius until analysis. Upon analysis, cells were centrifuged and the pellet washed until no precipitate remained. Next the pellet was resuspended in propidium staining (PI) solution containing 1 ml 10mg/ml Rnase A, 1 ml 10X PBS, 0.5ml 1 mg/ml PI, 10 ul 0.5 M EDTA, 10 ul Triton-X 100, brought to 10mls with water. Rnase A and PI were solubilized in citrate buffer containing 250 mM Sucrose, 40 mM sodium nitrate, and DMSO and adjusted at pH 7.6. Cells were kept on ice and brought to the Michigan State University's FACS facility and loaded into a flow cytometer for analysis. This approach measures DNA content of cells, which in turn provides a great deal of information about the cell-cycle. Specifically, cells in G_o/G_1 have 2n DNA, while cells in M have 4n DNA. Cells in S and G_2 have DNA content between 2n and 4n. Thus we used this application to determine the percentage of cells in each phase of the cell-cycle.

Statistical Analysis:

Some values were expressed as the mean +/- SE. Others were representative graphs pooled from 1 or 2 experiments where noted. Statistical differences between

values were examined by using the t-Test (two sample assuming unequal variances). The *p* values less than 0.05 were considered significant. Microsoft Excel was used for graphic design and data input. Microsoft Power Point was used for final graphs seen in this thesis. **Please note: "Images in this thesis are presented in color."**

RESULTS

Hypotheses I: NO stimulates osteoblast growth

Osteoblasts produce NO in response to mechanical strain and sex hormones suggesting that NO could be anabolic. Increases in bone formation can arise from increased osteoblast activity or increases in osteoblast number. To address NO production in our cells, we measured a stable-end product of NO, nitrite, in our medium. It has previously been shown that nitrite is directly proportional to NO derived from L-arginine.[42,62] Figure 7 shows that SNP treatment produces nitrite in a dose-dependent manner. To determine if nitric oxide could stimulate osteoblast growth, subconfluent (day 2) MC3T3-E1 mouse osteoblasts were treated with a nitric oxide donor, sodium nitroprusside (SNP). Twenty-four hours after treatment thymidine incorporation was measured. As shown in Figure 8A, treatment of osteoblasts with 10^{-4}M SNP results in a dramatic 13-fold increase in thymidine incorporation compared to untreated controls, while treatment of osteoblasts with 10^{-6} or 10^{-5}M SNP had no effect. To more carefully examine concentration dependence, osteoblasts were treated with SNP concentrations ranging between 10^{-5} and 10^{-4}M (Figure 8B,C). It is evident in Figure 8B that $2.5x10^{-5}$M greatly induced thymidine uptake 13-fold, so we further examined the concentration dependence of thymidine incorporation by using even smaller differences in SNP concentration. Figure 8C suggests that the critical SNP concentration required for thymidine incorporation ranges between 1.5×10^{-5} to 2×10^{-5}M.

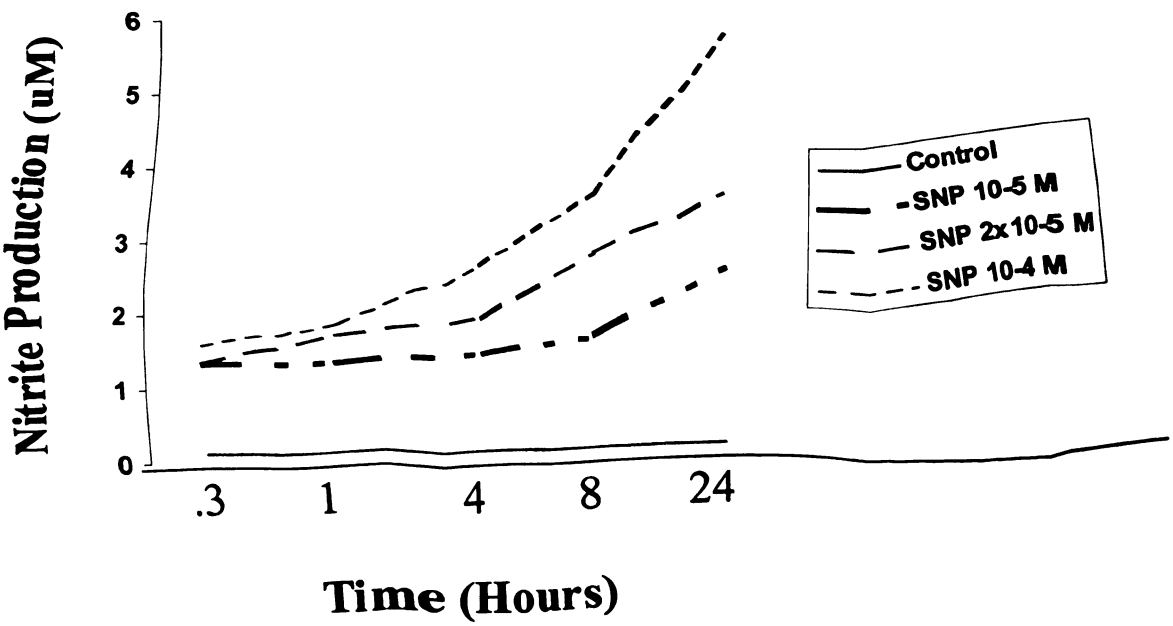

Figure 7 Nitrite production is dose-dependent under various sodium nitroprusside concentrations. On day 3, 100 ul of culture media was harvested from control or SNP-treated osteoblasts. Nitrite production was measured by addition of Griess Reagent 1 and 2 (50 ul of each), incubation for 10 minutes, and finally absorbance reading at 540nm. Values were normalized to a nitrite standard. Graph is representative of 2 experiments, each done in triplicate.

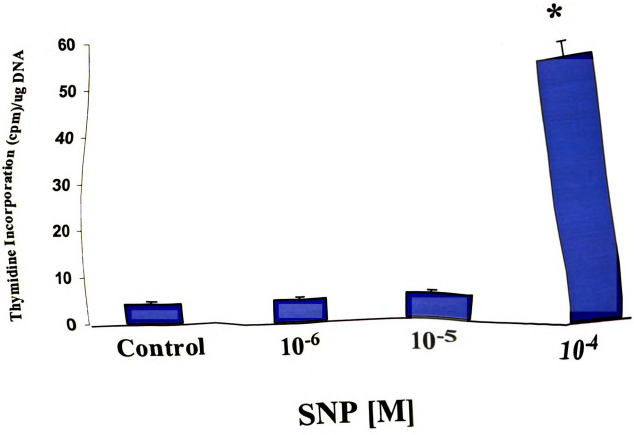

Figure 8A **SNP [10^{-4} M] induces thymidine incorporation in mouse MC3T3-E1 osteoblasts.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells per well in 6-well plates. The cells were fed 24 hours later, before treating with SNP [10^{-6} to10^{-4} M] on day 2. On day 3, cells were incubated with 4uCi/ml tritiated thymidine, and thymidine incorporation was measured via scintillation counting. Data is pooled from 5 experiments and expressed as averages +/- SE. *P<0.001.

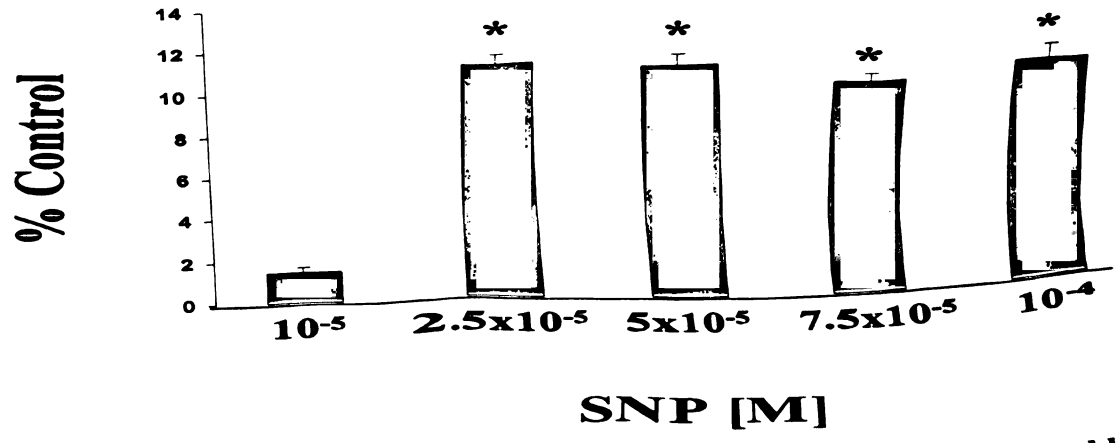

Figure 8B **SNP induces thymidine incorporation in mouse MC3T3-E1 osteoblasts in smaller concentrations as noted on graph.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells per well in 6-well plates. The cells were fed 24 hours later, before treating with SNP [10^{-6} to10^{-4} M] on day 2. On day 3, cells were incubated with 4uCi/ml tritiated thymidine and thymidine incorporation was measured via scintillation counting. Data is pooled from 3 experiments, each done in triplicate and expressed as averages +/- SE. *p<0.001.

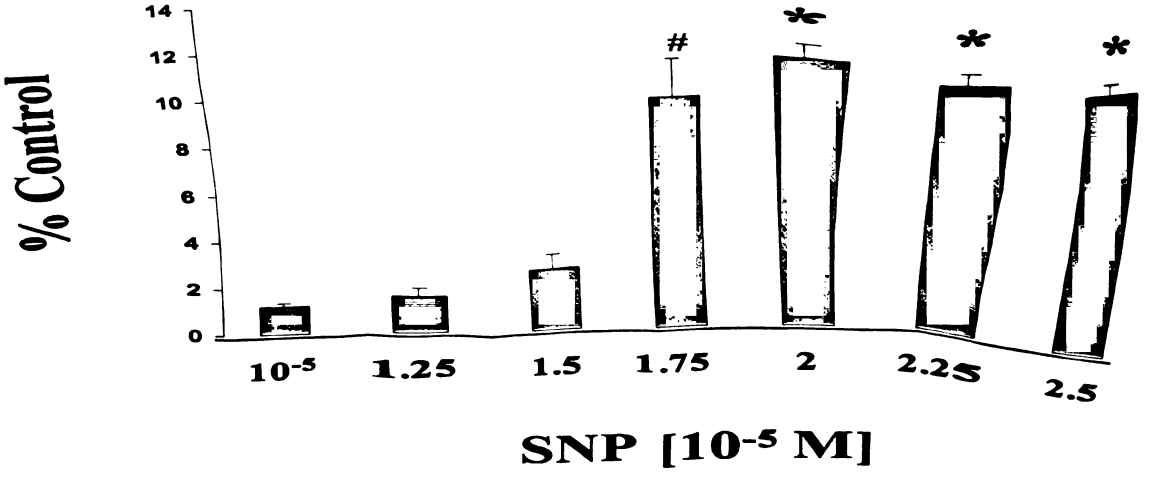

Figure 8C **SNP induces thymidine incorporation under even smaller concentrations noted on graph.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells and fed 24 hours later. On day 2, cells were treated with SNP [10^{-5}-$2.5x10^{-5}$ M]. On day 3, cells were incubated with 4uCi/ml tritiated thymidine for 2 hours before measuring thymidine incorporation via a scintillation counter. Data was pooled from 3 experiments and expressed as averages +/- SE. #p<0.002,*p<0.001.

NO savenger molecule

Nitric oxide donors have been shown to have effects that are not dependent on nitric oxide. To separate these effects from NO release, we treated osteoblasts with an NO scavenger, carboxy-PTIO. Figure 9 demonstrates that when carboxy-PTIO is added simultaneously with SNP, the SNP-induced thymidine incorporation is suppressed in a dose-dependent manner. Addition of carboxy-PTIO alone did not influence thymidine incorporation in control osteoblasts. These results suggest the SNP-induced thymidine incorporation is dependent on NO release. In addition, osteoblasts were treated with 20% serum, a known stimulator of osteoblast growth, to compare levels of thymidine incorporation to SNP levels. Figure 9 demonstrates a 2.5 fold increase in thymidine incorporation following serum stimulation, which is significantly lower than increases seen in response to SNP (10^{-4}M). Addition of carboxy-PTIO did not suppress this response (columns 8 and 9, Figure 9) thereby demonstrating that this compound does not have a general inhibitory effect on thymidine incorporation. .

Further analysis of cell growth

Induction of thymidine incorporation by SNP suggested that proliferation was significantly increased however, to our surprise, cell counts for days 3, 4, and 5 showed no significant difference between control and SNP treated osteoblasts (Figure 10). Corresponding with this finding, DNA measurements were also similar between conditions (Figure 11).

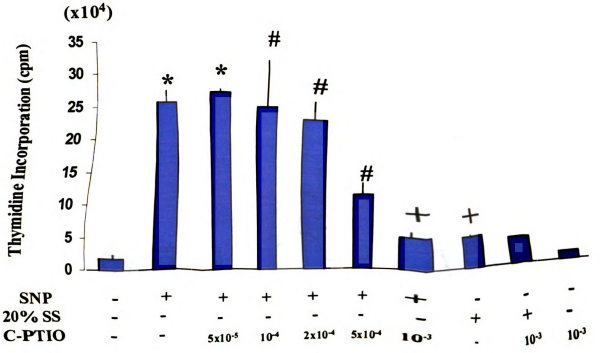

Figure 9 **carboxy-PTIO ablates the SNP induced thymidine incorporation in a dose-dependent manner.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells per well, in 6-well plates. The cells were fed 24 hours later and treated with SNP [10^{-4} M] and/or carboxy-PTIO [$5x10^{-5}$-10^{-3} M] on day 2. On day 3, cells were incubated for 2 hours with 4uCi/ml tritiated thymidine before harvesting and measuring thymidine incorporation via scintillation counting. Data are pooled from 3 experiments, each done in triplicate and expressed as averages +/-SE. *$p<0.001$, #$p<0.01$, +$p<0.05$.

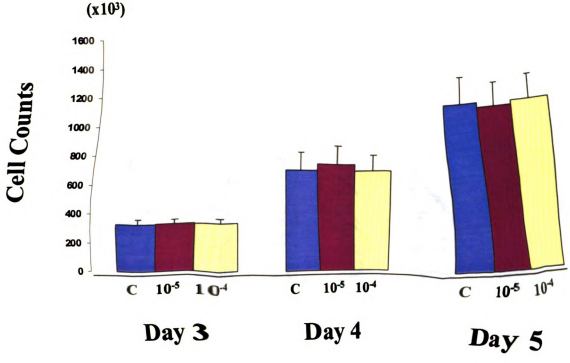

Figure 10 **SNP does not influence cell number in a dose-dependent manner in day 3, 4, & 5 mouse osteoblasts.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells per well, in 6-well plates. The cells were fed 24 hours later, and treated on day 2 with SNP [10^{-5} and 10^{-4} M]. On day 3,4 and 5 cells were trypsinized and counted on a hemocytometer. Data are pooled from 5 experiments, each done in triplicate and expressed as averages +/- SE.

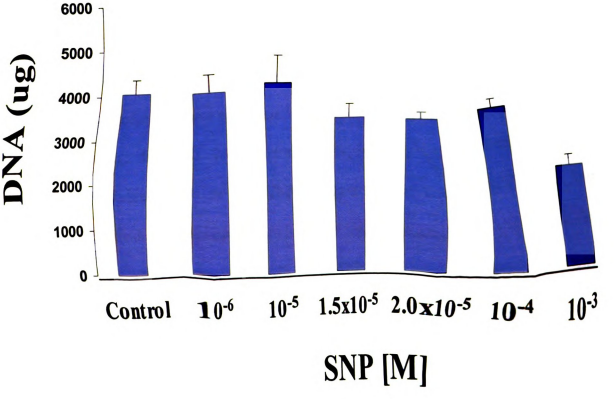

Figure 11 **DNA measurements using Hoescht dye analysis.** MC3T3-*E1* osteoblasts were plated at $4x10^4$ cells per well, in 6-well plates. The cells were fed 24 hours later, and treated on day 2 with SNP [10^{-6} and 10^{-3} M]. DNA measurements were calculated using fluorometric analysis. (As described in Methods Section)

Hypothesis II: NO promotes osteoblast death

One possible explanation for our observation that thymidine incorporation is increased without a matched increase in cell number is that elevated rates of proliferation are matched by elevated levels of cell death, either by necrosis or apoptosis. To test this hypothesis we included trypan blue staining in cell counts to determine the percentage of viable cells in treated and untreated conditions. Figure 12 demonstrates that only high concentrations ($>10^{-3}$M) of SNP significantly reduced cell viability. These findings are further supported by the lack of a significant change in LDH activity in the media Figure 13. The next possibility is that apoptosis is increased. This would be consistent with reports demonstrating that NO has apoptotic activity in a variety of cells. Figure 14 illustrates some characteristics of TNFα-induced apoptotic MC3T3-E1 mouse osteoblasts. TNFα-induced apoptotic cells were then compared to propidium iodide fragmented nuclei to verify apoptotic cells. However, when apoptotic cell death was examined by propidium iodide staining, the number of cells containing nuclear fragmentation did not differ between control and SNP treated cells at 24 and 48 hours after treatment (Figure 15). Figure 16 illustrates some representative microscopic images of propidium iodide staining in osteoblasts.

Thymidine kinase measurements

Thymidine incorporation into DNA requires its phosphorylation by thymidine kinase, therefore an increase in thymidine kinase activity could account for increased radiolabeled thymidine being incorporated per unit DNA. However, measurement of

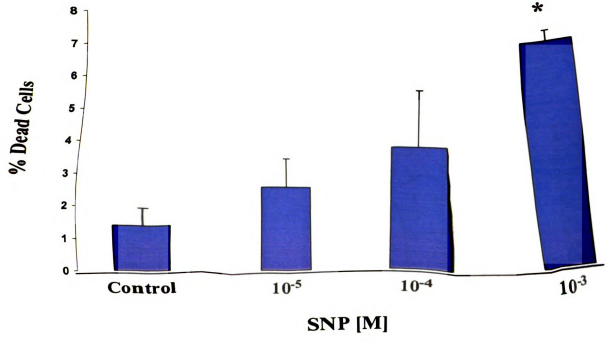

Figure 12 **SNP [10^{-3} M] significantly reduces cell viability in MC3T3-E1 mouse osteoblasts.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells and fed 24 hours later. On day 2, cells were treated with SNP [10^{-5}-10^{-3} M]. On day 3, cells were trypsinized and counted on a hemocytometer. Trypan blue 0.4% (4mg/ml) was added to cell mix to determine the percentage of viable cells excluding trypan blue stain. Data was pooled from 5 experiments, each done in triplicate and expressed as averages +/- SE. *p<0.001.

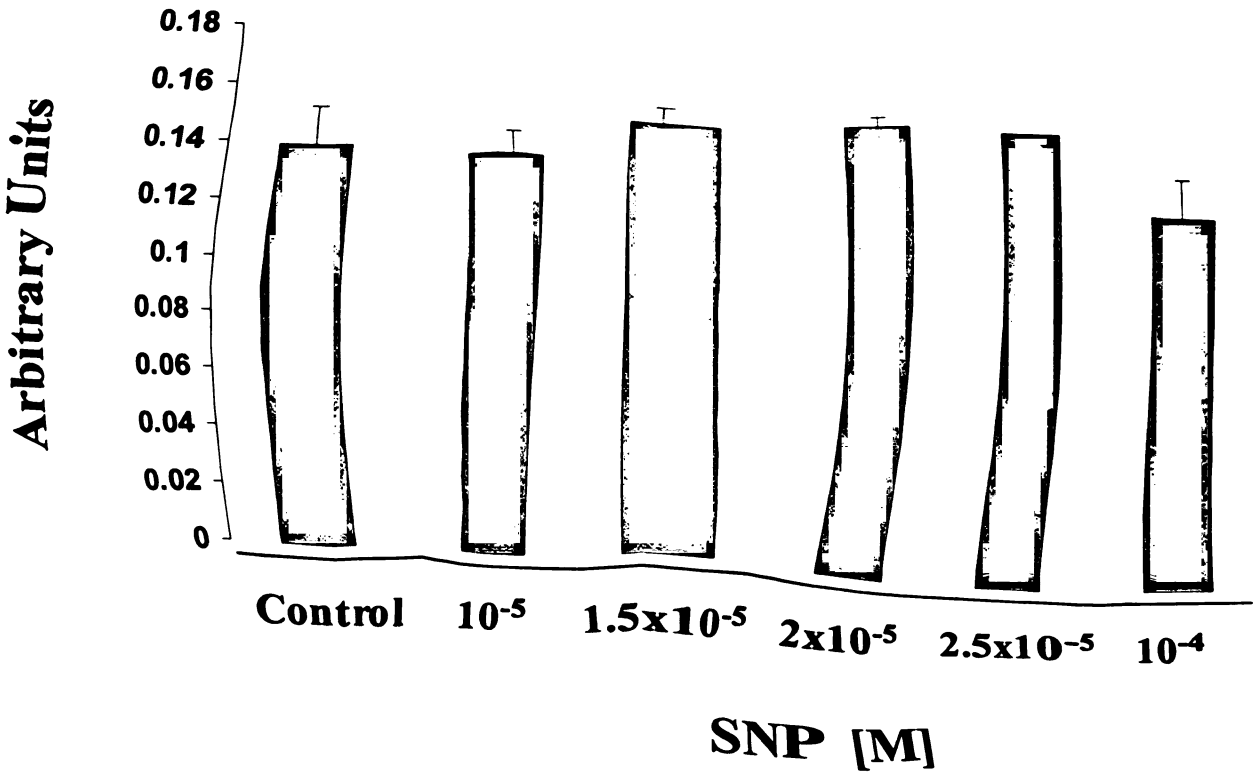

Figure 13 **Lactate dehydrogenase (LDH) measurements in SNP-treated osteoblasts.** MC3T3-E1 osteoblasts were plated at $4x10^4$ cells in 6-well plates and fed 24 hours later. On day 2, cells were treated with SNP [10^{-5}-10^{-4} M]. Samples of media were collected on day 3 and LDH measurements were calculated as described in Methods section.

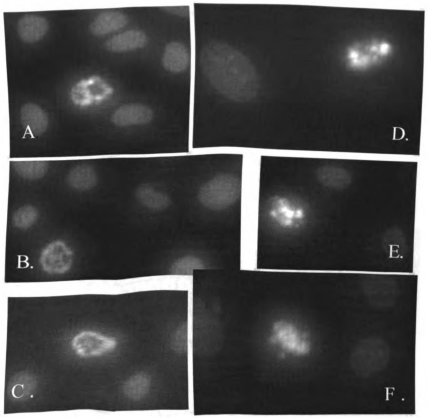

Figure 14 **Characteristics of TNFα-induced apoptosis in MC3T3-E1 mouse osteoblasts.** MC3T3-E1 osteoblasts were plated at $1x10^4$ cell density in 12-well plates and fed 24 hours later. On day 2, cells were treated with 20ng/ml TNFα before examining 24 hours later. A-C. shows perinuclear DNA in osteoblasts representive of early apoptosis, D,E. shows highly condensed DNA in osteoblast nuclei representative of later apoptosis, while F. is characteristic of end stage apoptosis by the presence of apoptotic bodies.

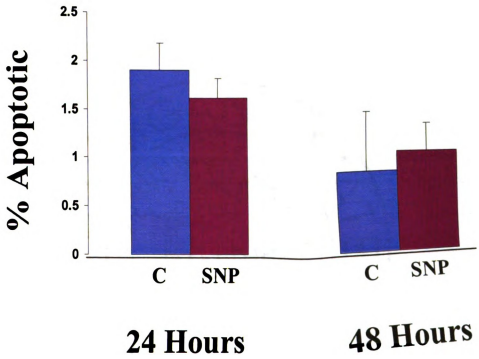

Figure 15 SNP [10^{-4} M] does not induce apoptosis in MC3T3-E1 mouse osteoblasts over 48 hours. MC3T3-E1 osteoblasts were plated at $1x10^4$ cells per well in 12-well plates and fed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M], before examining 24 and 48 hours later by propidium iodide staining. Fragmented nuclei were counted in 12 random fields, then compared to 20ng/ml TNFα-induced apoptotic cells to verify apoptotic cells. Data is pooled from 3 experiments, each done in triplicates and expressed as averages +/- SE.

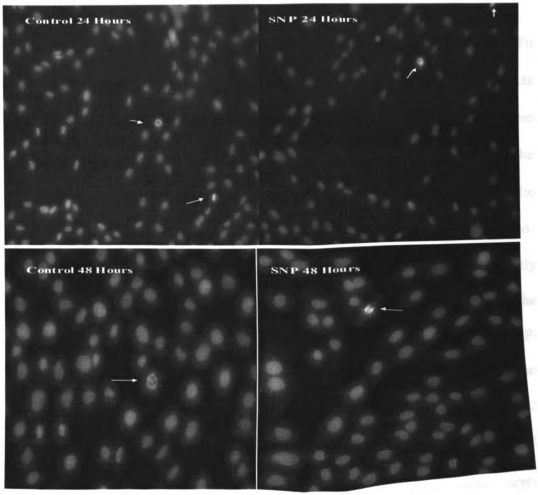

Figure 16 Propidium iodide staining of osteoblast nuclei in control and SNP [10^{-4} M] conditions at 24 (A,B) and 48 hours (C,D). Arrows are indicative of apoptotic cells verified by comparing to 20ng/ml TNFα-induced apoptosis in osteoblasts as shown in Figure 14. To confirm cells were not dividing and were in fact apoptotic, cells were viewed under bright field.

thymidine kinase activity showed no significant changes between conditions (C-17,652 +/-1387; SNP 17,749 +/-838, n=2).

Thymidine localization

Thymidine can be incorporated into nuclear or mitochondrial DNA. To distinguish between these compartments, we isolated nuclear and whole cell extracts following a 2-hour treatment with tritiated thymidine, and measured thymidine incorporation in the presence or absence of SNP. Figure 17 demonstrates that most of the thymidine uptake occurs in the nucleus. SNP-induced thymidine incorporation into the nuclear fraction was ablated by the addition of carboxy-PTIO (data not shown). Bromo-deoxyuridine (Brd-U) incorporation (Figures 18 and 19) further verifies a predominantly nuclear localization of new DNA synthesis in SNP treated versus untreated cells. The percentage of cells with Brd-U incorporation was similar between control and SNP-treated conditions (Table I). However, Brd-U staining of cell nuclei showed more intensive staining in SNP treated cells compared to the control (Table II). Figure 20 illustrates different images of BrdU incorporation in the nuclei of osteoblast cells. Starting at the upper left of the illustration, a highly fluorescent positive Brd-U labeled nuclei, to a weakly positive (lower fluorescent) Brd-U nuclei in the lower right corner. Lastly, Figure 21 analyzes nuclei from control and SNP-treated ostoblast. Nuclei were grouped into four classes based on localization of Brd-U incorporation. Group I is highly fluorescent nuclei with no foci present. Group II contains weaker fluorescent nuclei with no foci present. Group III displays few foci (<15) compared to group IV which contain many unequally distributed foci (>15). Osteoblasts treated for 24 hours with SNP had a

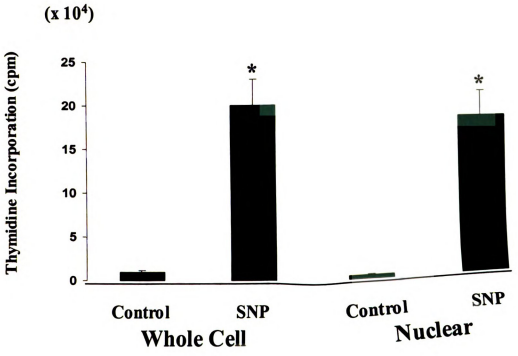

Figure17 SNP-induced thymidine incorporation is nuclear. MC3T3-E1 osteoblasts were plated at $4x10^4$ cells and fed 24 hours after plating. On day 2, cells were treated with SNP [10^{-4} M]. On day 3, cells were incubated with 4uCi/ml tritiated thymidine for 2 hours, before harvesting cells. Whole cell thymidine incorporation was determined as mentioned in methods section, while the nuclear fraction was isolated via washing cells with NP40 lysis buffer and nuclei verified by trypan blue staining. Data was pooled from 3 experiments, each done in triplicates and expressed as averages +/- SE. *$p<0.01$.

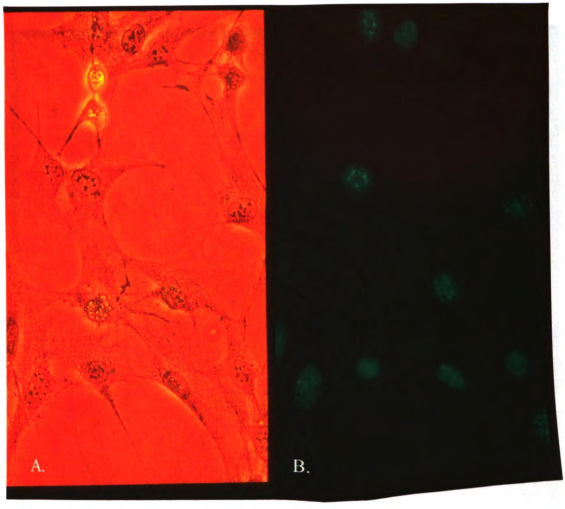

Figure 18 Brd-U incorporation in control osteoblast nuclei. Phase contrast image of control nuclei (A), and Brd-U stained nuclei (B). Osteoblasts were plated at a density of $4x10^4$ cells and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M] before treating with 10uM Brd-U on day 3 for 2 hours. Cells were examined by a reflective light fluorescent microscope (as described in Methods).

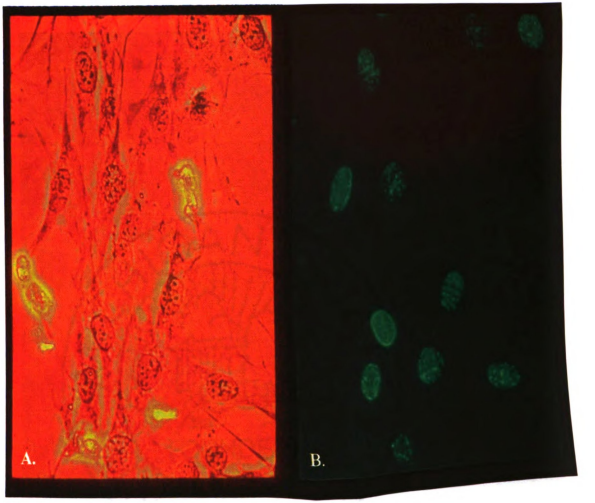

Figure 19 **Brd-U incorporation in SNP treated osteoblast nuclei.** Phase contrast image of nuclei (A), and Brd-U stained nuclei (B). Osteoblasts were plated at a density of $4x10^4$ cells and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M] before treating with 10uM Brd-U on day 3 for 2 hours. Cells were examined by a reflective light fluorescent microscope (as described in Methods).

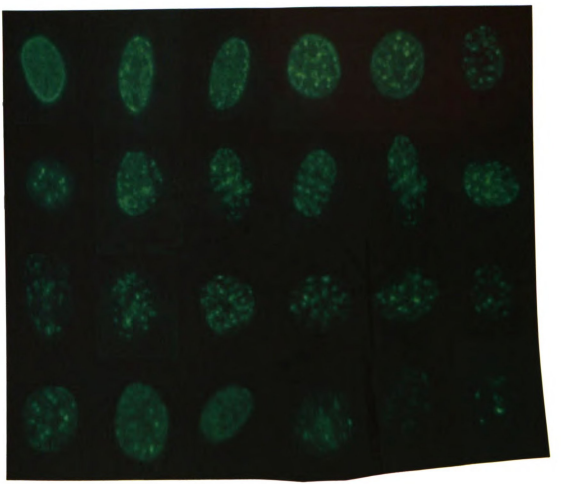

Figure 20 **Nuclear Brd-U patterns in osteoblast nuclei visualized by indirect immunoreactivity (FITC).** Osteoblasts were plated at a density of $4x10^4$ cells and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M] before treating with 10uM Brd-U on day 3 for 2 hours. Cells were examined by a reflective light fluorescent microscope (as described in Methods).

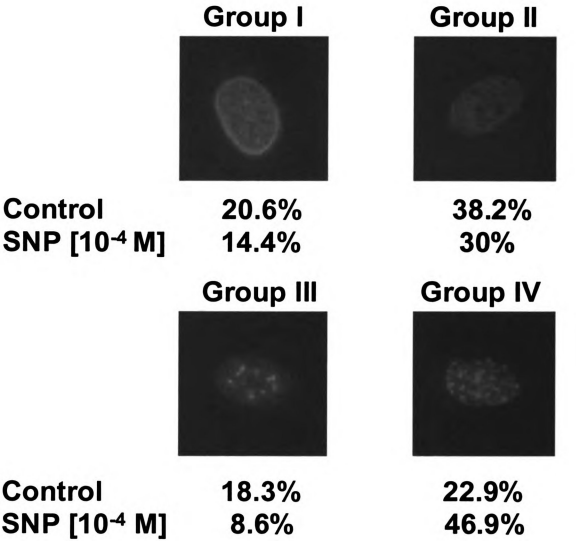

Figure 21 **Brd-U analysis of control and SNP [10^{-4} M] treated osteoblasts.** Osteoblasts (day 2) were treated for 24 hours with or without SNP and pulsed for 2 hours with Brd-U. Nuclei were grouped into four classes (Group I, II, III, & IV) based on Brd-U incorporation pattern. Group I represents highly fluorescent nuclei with no nuclear foci. Group II represents weakly fluorescent nuclei with no nuclear foci. Group III display few (<15 foci) nuclear foci. Group IV display more (>15) nuclear foci relative to group III. Osteoblast nuclei with similar DNA distribution characteristics relative to particular group were scored for that group. Final percentages were determined by analyzing nuclei pooled from two separate experiments. Total nuclei counted in control condition were 471 and SNP-treated were 603.

	% Brd-U	% +/-
Control	**45.5**	**2.1**
SNP [10^{-4} M]	**47.7**	**1.6**

Table I **Percent Brd-U positive cells with and without SNP [10^{-4} M] treatment.** Osteoblasts were plated at a density of $4x10^4$ cells on cover slips in 6-well plates and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M] before treating with 10uM Brd-U on day 3 for 2 hours. Cells were examined by a reflective light fluorescent microscope (as described in Methods). Percentage of positive Brd-U cells were calculated for each condition. Data was pooled from 3 experiments (700cells/experiment) and expressed as averages +/- SE.

	Intensity	+/-
Control	**49.9**	**2.04**
SNP [10^{-4} M]	**56.0**	**2.23**

Table II **Intensity of Brd-U Incorporation relative to Background (0).** Osteoblasts were plated at a density of $4x10^4$ cells on cover slips in 6-well plates and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M] before treating with 10uM Brd-U on day 3 for 2 hours. Cells were examined by a reflective light fluorescent microscope, and the intensity of fluorescence was determined as described in Methods. Data was pooled from 3 experiments and expressed as averages +/- SE.

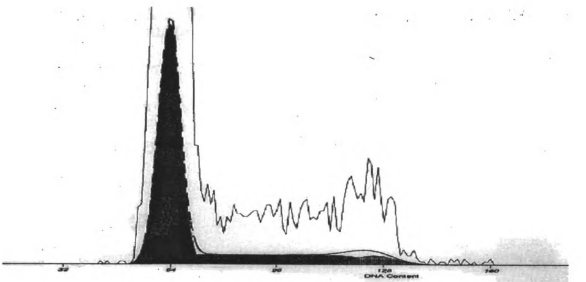

Figure 22 Cell-cycle illustration of osteoblast cells in normal replicative growth and in response to SNP treatment [10^{-4} M]. This diagram is constructed during FACS analysis. From left to right of figure, large G_1 phase (left) to S-phase (middle), G_2/M-phase (right).

Cell-Cycle Phase	% G_1	% S	% G_2
Control	64.9	23.8	11.3
SNP [10^{-4} M]	69.1	20.4	10.5

Table III Flow Cytometric Analysis (FACS). Osteoblasts were plated at a density of $4x10^4$ cells and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M] and FACS analysis was done on day 3 (as described in Methods). The table represents the phase of the cell-cycle that treated and non-treated osteoblasts were found in. Data is pooled from 2 separate experiments each done in triplicate.

higher percentage of Group IV type nuclear patterns compared to control (46.9% vs.22.9%). In contrast, Group III-like nuclear pattern was marked by a decrease compared to control (8.6% vs. 18.3%). Our finding of increased nuclear Brd-U with SNP treatment is consistent with nuclear patterns with DNA repair as shown previously demonstrated in other systems.[131-133]

Flow Cytometry (FACS)

Flow cytometry (FACS) was done to determine whether a cell-cycle block was occurring causing the increased thymidine incorporation in SNP treated cells (Table III). FACS data demonstated that there was no cell-cycle block in SNP treated cells, in fact, treated and untreated conditions showed similar percentages of cells in each phase of the cell cycle. Furthermore, it was interesting to find that at 48 hours after SNP treatment, thymidine incorporation was only 3-fold that of the control (Figure 23). The fact that thymidine incorporation was reduced after 48-hours supports a possible DNA repair event may have occurred.

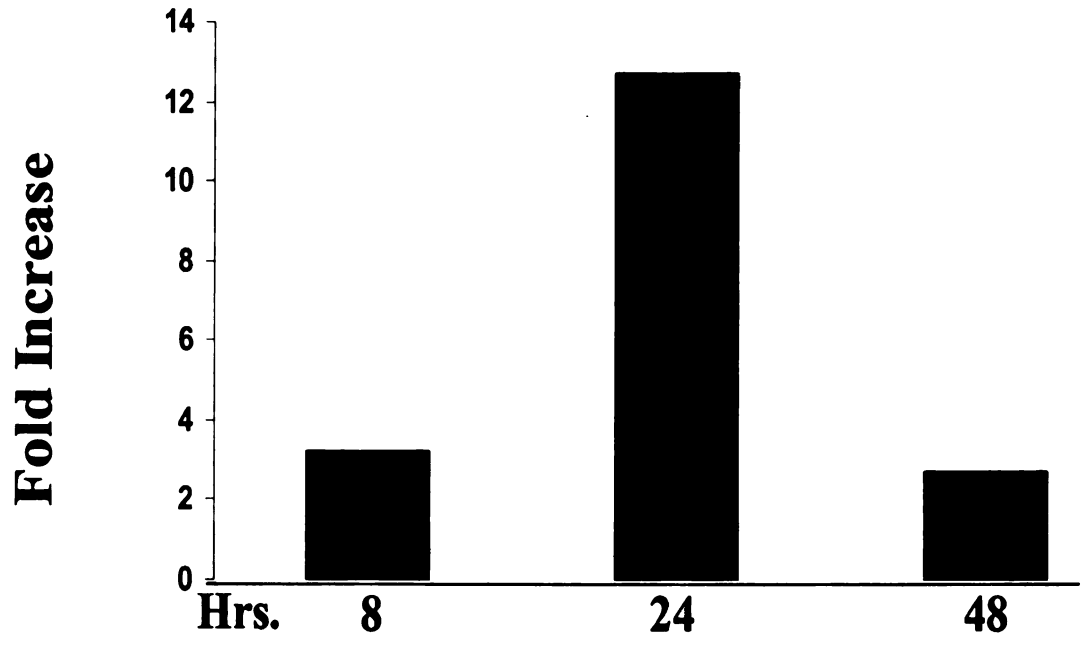

Figure 23 Thymidine Incorporation in MC3T3-E1 osteoblasts at 8, 24 and 48 hours after SNP-treatment [10^{-4} M]. Cells were plated at $4x10^4$ in 6-well culture plates and refed 24 hours later. On day 2, cells were treated with SNP [10^{-4} M]. On day 3, cells were incubated with 4uCi/ml tritiated thymidine and thymidine incorporation was measured via scintillation counting. This data represents 1 experiment done in triplicate (8 & 48 hours) and data pooled from 5 experiments done in triplicate (24 hours).

DISCUSSION

Nitric oxide levels are increased in bone in response to anabolic conditions such as mechanical strain and estrogen treatment. This suggests that NO could be anabolic. Therefore, we examined the influence of NO on osteoblast growth and subsequently death. Our most significant finding is that thymidine incorporation is markedly increased in response to treatment with a nitric oxide donor, SNP [10^{-4} M]. Co-treatment with an NO scavenger demonstrates that this effect is dependent upon NO. Several reports have suggested that NO can stimulate cell proliferation. Specifically, Kanamaru et al. reported that SNAP induced DNA synthesis of MC3T3-E1 *in vitro* by measuring thymidine incorporation.[122] However cell numbers were not measured. Riancho et al showed that when endogenous NO production is inhibited, osteoblast growth decreases and is restored with the addition of SNP [10^{-4} M].[68] Similar to our results, it is reported that SNP significantly induced incorporation of [H^3] thymidine in L929 fibroblasts suggesting that NO is a positive factor in fibroblast proliferation.[123] Although they inferred that proliferation was increased, cell counts were not done. In fact several reports of NO treatment increasing proliferation in other systems also did not count cells. These findings support our data indicating that thymidine induction was greatly induced following SNP [10^{-4} M] treatment, and do not contradict our cell count data because these earlier studies did not perform cell counts.

Some reports even suggest a growth inhibitory effect of NO.[87,92] In MC3T3-E1 cells, SNAP [10^{-3} M] is reported to reduce cell number throughout 48-hours. Human

osteoblasts treated with an inhibitor of NOS showed no change unless treated with cytokines in which case the NOS inhibitor partially inhibited their growth inhibitory effects, suggesting a negative role of NO on growth.[87] Moreover, it has been shown that individual cytokines were able to significantly decrease thymidine incorporation in human osteoblasts.[87]

It is also reported that SNP induces damage in rat osteoblasts, ROB and ROB-C26 cells.[87] It has been speculated that the cytotoxic effect of SNP may be due to the cyanide component it generates upon decomposition. This may be producing non-physiologic effects on cells. We addressed this concern by using an NO scavenger (carboxy-PTIO) to determine whether the changes in thymidine incorporation were NO mediated or due to some byproduct of the SNP donor molecule. We conclude that it is an NO effect and not due to a by-product of the donor molecule. Furthermore, Chae et al. addressed this concern by treating ROS 17/2.8 cells with potassium ferrocyanide since cyanide is a byproduct of SNP.[88] They reported that SNP [$3x10^{-5}$ M] elevated alkaline phosphatase activity after 48 hours of treatment in differentiating rat osteoblasts. The same concentration of potassium ferricyanide did not show any effect on the biological activity of alkaline phosphatase in ROS 17/2.8 cells. This suggests that cyanide does not play a role in the biological effects induced by SNP.

The observation of induced thymidine incorporation without cell growth suggests that NO may stimulate proliferation while at the same time stimulating cell death. Nitric oxide has been reported to cause a necrotic effect in cells.[124] Specifically, donors used

(SNAP) at concentrations greater than $0.5x10^{-4}$ M produced a significant cytotoxic effect over a 48-hour period. Our findings demonstrate that there is a dose-dependent decrease in trypan blue exclusion, with no significant change in LDH measurements. In support of this, it has also been reported that SNAP reduces dose-dependent trypan blue exclusion in a dose-dependent manner, signifying reduced cell viability, but showed no change in LDH measurements.[87]

NO has also been demonstrated to be an important mediator of apoptosis. There are many reports that NO ($>10^{-4}$ M) damages cells and causes cell death via apoptosis. This has been elegantly shown in the studies of Mogi et al. who demonstrated that cytokine (IL-1, TNFα, & INF-γ) induced NO production, causes a potent decrease in MC3T3-E1 osteoblast cell proliferation and eventually induces apoptosis.[100] They also reported that exogenous NO production via SNAP [10^{-4} M] caused DNA fragmentation greater than that seen using the cytokine mixture. Contrary to this study, we showed that SNP [10^{-4} M] did not increase apoptosis through 48 hours of treatment, although apoptosis was evident at SNP [10^{-3} M] (data not shown). Differences between these findings could be a result of differences in NO donors used. Specifically, SNAP releases NO into the medium at a much faster rate[87] than SNP. This would cause larger NO concentrations than SNP over a given period of time. Moreover, it was demonstrated that NO donors SNAP, GSCN, and SIN-1 at [10^{-4} M] did not affect the viability of MC3T3-E1 osteoblasts.[44]

We report that most of the thymidine incorporation was in fact nuclear versus cytosolic. This was confirmed by bromodeoxyuridine (Brd-U) incorporation into cell nuclei. Intranuclear dispersion of BrdU tagged fluorescent intensities allows discrimination between positive and negative BrdU cells.[125] SNP-treated cells had a similar percentage of Brd-U positive cells as untreated cells, however Brd-U was clearly more intense in SNP-treated versus control cells. In addition, we noted differences in Brd-U incorporation patterns within the nucleus. Our main focus is DNA distribution and intensity, and how this relates to cell growth and DNA repair. The main changes from the beginning to the end of the S phase generally concern size of spots (small at the beginning, large at the end of S phase), the spot number, and location of spots relative to nuclear and nucleolar boundaries. Early replication is characterized as small spot size, whereas, late replication is characterized by patterns of perinuclear labeling and large spot size.[126] Large spots are generally indicative of heterochromatin,[127] but they may also represent mitotic figures,[128] increased rate of synthesis,[129] larger portion of AT-rich sequences,[130] or simply larger quantities of DNA.[264] The speckled-like appearance of DNA in the Brd-U incorporation (Group IV in figure 21) in the SNP-treated condition may be this repair process fixing the DNA. We speculate this because SNP group IV is about 24% higher than the control condition. In support of this, Tomalin et al. reported a strikingly similar nuclear pattern when they induced DNA repair synthesis with bleomycin (radiomimetic drug) and irradiation in primary human skin fibroblasts.[131] Furthermore, other investigators visualizing expression of DNA repair proteins in the nucleus during the repair process are finding their presence in discrete foci associated with sites of DNA repair.[132,133]

Flow cytometry (FACS) demonstrates that SNP-treatment did not influence cell-cycle progression. FACS also confirmed that apoptosis was not evident in either the SNP-treated or control conditions. Because osteoblast number or cell death (necrosis or apoptosis) were unaffected by SNP [10^{-4} M], it is possible that the significant induction of thymidine incorporation is due to DNA repair. It was interesting to find that at 48 hours after SNP treatment, thymidine incorporation was only 3-fold that of the control (Figure 23). The fact that thymidine incorporation was reduced after 48-hours supports a possible DNA repair event may have occurred. It may be that NO induces DNA damage, thus eliciting DNA repair. DNA polymerase is an enzyme involved in DNA repair. Therefore, to test if DNA repair is causing an increase in thymidine uptake, MC3T3-E1 cells could be treated with Lithocholic acid (a selective inhibitor to DNA polymerase beta, which is involved in repair, but not in general transcription) in the presence and absence of SNP. We would expect the percentage of nuclei with the pattern of Brd-U labeling evident in group IV to be lower.

To speculate on the means of DNA repair it is known that nitrites may deaminate cytosines particularly to uracil. Aforementioned, these uracil bases are quickly replaced by thymine bases. It is interesting to note, that the thymidine incorporation correlates with nitrite production by sodium nitroprusside. It may be that nitrite is causing deamination to cytosine bases, thus the increased thymidine incorporation is due to the excision repair process.

It is evident that we have identified the concentration or *threshold of NO that may* activate the DNA repair system in osteoblasts. The exact *mechanism by which* this system is activated remains to be elucidated. This is supported by the investigations by other authors that used NO donors, showing that higher concentrations of NO cause cell damage, whereas, lower concentrations seem to benefit the activity and function of osteoblasts. This is consistent with other NO affects that show dose-dependence such as that reported in Damoulis and Hauschka (1997) and Kanamaru et al. (2001).

Our findings are important to the bone research field because we provide insight into the effects of NO on osteoblasts. There are many possibilities in which NO may be involved in anabolic and catabolic effects on bone. Our results demonstrate a dose-dependency in these responses, but also potentially an intermediate effect. We speculate that at threshold concentrations of NO before cell death occurs, osteoblasts may be undergoing DNA repair in response to DNA damage caused by NO. As levels of NO increase, nitric oxide has the ability to inhibit DNA repair enzymes as well as cause lesions in DNA, which may be contributing to cell death. This method of cellular demise may be one of the contributing factors evident in arthritic bone loss. Further research will need to focus on whether NO causes DNA damage in osteoblasts and what repair systems may be involved. Elucidating these effects may provide better therapeutic approaches to prevent bone loss seen in inflammatory conditions and lead to greater understanding of basic biological effects of NO on cell systems.

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