UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Defective NO/cGMP/PKG II Signaling in Diabetic Osteoblasts /

Permalink

https://escholarship.org/uc/item/1b26j3dh

Author

Chan, Geraldine Cawah

Publication Date

2013

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Defective NO/cGMP/PKG II Signaling in Diabetic Osteoblasts

A Thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Geraldine Cawah Chan

Committee in Charge:

Professor Renate Brigitte Pilz, Chair
Professor Richard A. Firtel, Co-Chair
Professor Mark Andrew Estelle

2013

The thesis of Geraldine Cawah Chan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego
2013

TABLE OF CONTENTS

Signature Page iii

Table of Contents iv

List of Figures v

Acknowledgements vi

Abstract vii

Introduction 1

Materials and Methods 7

Results 13

Discussion 17

Figures 21

References 29

LIST OF FIGURES

Figure 1: Characterization of Murine Primary Osteoblasts. 21

Figure 2: Decreased NO production and reduced eNOS-Hsp90α association in diabetic osteoblasts. 22

Figure 3: Decreased PKG II expression in diabetic osteoblasts 23

Figure 4: Hyperglycemia impedes Akt and ERK activation by NO..... 24

Figure 5. Defective ERK/Akt signaling in diabetic osteoblasts 25

Figure 6: Effect of Cinaciguat on VASP activation and Fos family gene expression..... 26

Figure 7: cGMP and Cinaciguat effectively induces ERK and Akt phosphorylation in control and diabetic osteoblasts 27

Figure 8: Cinaciguat increases osteoblast proliferation..... 27

Figure 9: Defective NO/cGMP/PKG II Signaling in Diabetic Osteoblasts..... 28

ACKNOWLEDGEMENTS

Foremost, I would like to offer my special thanks to my advisor Dr. Renate Pilz for her support and constant enthusiasm. I am thankful for her advice and encouragement throughout my time at the lab. I would also like to thank Dr. Gerry Boss for his advice in the making of this thesis and my presentation.

I would also like to offer my special thanks to Dr. Richard Firtel, Dr. Michael David, and Dr. Mark Estelle for being members of my committee.

I would like to express my very great appreciation for Dr. Hema Kalyanaraman for her support and guidance throughout this project. Without her, this would not have been possible. Furthermore, I am grateful for her patience throughout the many edits of this thesis.

I wish to thank Dr. Wolfgang Dillman and Brian T. Scott for help in generating STZ-induced Type I diabetic mice

Last but not least, I would like to thank all members of the Boss-Pilz Lab for all their guidance and moral support these past two years.

Materials and Methods, in part is currently being prepared for submission for publication of the material. Pilz, Renate B; Kalyanaraman, Hema; Chan, Geraldine C; Scott, Brian T; Dillman, Wolfgang. The dissertation author was the primary investigator and author of this material.

Figures, in part is currently being prepared for submission for publication of the material. Pilz, Renate B; Kalyanaraman, Hema; Chan, Geraldine C; Scott, Brian T; Dillman, Wolfgang. The dissertation author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Defective NO/cGMP/PKG II Signaling in Diabetic Osteoblasts

by

Geraldine Cawah Chan

Master of Science in Biology

University of California, San Diego, 2013

Professor Renate Brigette Pilz, Chair
Professor Richard A. Firtel, Co-Chair

Osteoporosis is a lesser known complication of diabetes mellitus type I (DM1). Patients with DM1-associated osteoporosis experience increased fracture risk and delayed fracture healing, resulting from decreased bone formation while bone resorption remains unchanged. However little is known about the mechanism of the diabetic bone phenotype. Our lab has shown that the NO/cGMP/PKG II pathway is highly involved in osteoblast mechanotransduction and estrogen induced osteoblast proliferation and survival. We therefore hypothesized that defects in the NO/cGMP/PKG II signaling may contribute to the pathology of diabetes associated osteoporosis.

Multiple points within the signaling pathway were assessed with osteoblasts isolated from control and streptozotocin (STZ) induced DM1 mice to elucidate alterations due to diabetes. Immunoprecipitation of eNOS showed decreased eNOS-Hsp90α association, suggesting lower eNOS activity confirmed by reduced NO production in diabetic osteoblasts. Cytosolic PKG I levels were unaltered and membrane-bound PKG II showed decreased expression in diabetic osteoblasts. Further defective Erk/Akt signaling and reduced sensitivity to the NO donor, PAPA NONOate was also observed under hyperglycemic conditions. We also explored if cGMP-elevating agent cinaciguat was effective in enhancing PKG activity and the downstream signaling. Treatment of diabetic osteoblasts with cinaciguat showed increased VASP phosphorylation, Erk/Akt activation and fos family gene expression. Further, bromodeoxyuridine (BrDU) labeling of diabetic osteoblasts treated with cinaciguat effectively increased osteoblast proliferation. In this project, we found that the NO/cGMP/PKG signaling is impaired in diabetic osteoblasts and cinaciguat may be effective in treating osteoporosis in patients with type 1 diabetes.

INTRODUCTION

Diabetes mellitus (DM), characterized by high blood glucose levels, results from insufficient insulin production or cellular insensitivity to insulin— defining DM Type 1 (DM1) and DM Type 2 (DM2) respectively. Type I diabetic patients often show signs of micro-vascular complications like retinopathy, neuropathy, and nephropathy. However, one lesser known complication observed in diabetic patients is osteoporosis resulting in increased fracture risk and delayed fracture healing (Espallargues, 2001). As the average life expectancy increases and more people are diagnosed with diabetes, age-related frailty and impaired vision promotes a larger number of falls leading to fractures (Hamann, 2012).

Bone remodeling is a balanced process between osteoclasts and osteoblasts, bone cells responsible for bone resorption and formation respectively. The diabetic skeletal phenotype is characterized by reduced osteoblastic bone formation and low bone mineral density (BMD) in the presence of normal osteoclast number and function (Verhaeghe, 1997; Waud, 1994). Rapid bone loss and increased fracture risk has been observed in both pharmacologic (streptozotocin-induced) and genetically predisposed rodent models of type I diabetes (Botolin, 2006). Although DMII patients exhibit equivalent or increased BMD compared to age matched controls, they experience increased fracture risk due to poorer bone quality (Schwartz, 2004). Although several studies have been performed in diabetic rodent models, the molecular mechanism that contributes to the bone pathology in diabetic patients remains understudied.

There are two main theories for decreased osteoblast activity observed in DM1. First, it is hypothesized that increased bone marrow stromal cells differentiate into

adipocytes rather than osteoblasts. When cells commit to adipocytes, peroxisomal proliferator-activated receptor (PPARγ) expression hinders osteoblast differentiation, proliferation, and maturation. Together, these factors lead to lower numbers of mature active osteoblasts and thus decreased bone formation (Thrailkill, 2005) Second, hyperglycemia elevates advanced glycation end-product (AGE) formation which in turn increases oxidative stress on bones through intracellular reactive oxygen species (ROS) generation. Oxidative stress inhibits osteoblast differentiation while it also induces osteoblast apoptosis, contributing to lower bone formation (Hamada, 2009). AGE has also been shown to modulate various genes involved in bone metabolism where AGE accumulation suppresses osteoblast and promotes osteoclast activities, thereby shifting the balance towards bone resorption (Franke, 2011). AGE also disrupts collagen cross-linking leading to collagen stiffness and thus bone fragility (Blakytny, 2011). However the cellular signaling events leading to the diabetic bone phenotype remains unclear.

Nitric oxide (NO) plays an essential role in bone metabolism, increasing and lowering osteoblast and osteoclast activity respectively. Strict regulation of NO levels is necessary for proper bone homeostasis. The main source of NO in osteoblasts and osteocytes is endothelial NOS (eNOS) (MacPherson, 1999). Targeted deletion of eNOS in mice results in profound bone abnormalities, reduced mineral apposition rate and blunted response to estrogens (Armour, 2001). While low NO concentrations may increase osteoblast cell proliferation and activity, higher concentrations promotes osteoblast apoptosis and bone resorption (Brandi, 1995). NO activates soluble guanylyl cyclase (sGC) to generate cyclic GMP that activates both soluble type I and membrane-bound type II cGMP dependent protein kinases (PKG). cGMP is also synthesized by

receptor guanylyl cyclase (rGC) upon naturietic peptide stimulation. Previous studies in our laboratory have shown a crucial anabolic role of the NO/cGMP/PKG II signaling cascade in osteoblast proliferation and survival (Marathe, 2012; Rangaswami, 2010; Rangaswami, 2012). Mechanical stimulation (e.g. fluid shear stress) of osteoblasts leads to NO/cGMP/PKG II mediated activation of ERK and Akt resulting in increased osteoblast proliferation.

Several studies have shown that estrogen deficiency found in post-menopausal women directly correlates with bone integrity (Riggs, 1982; Ebling, 1996). Furthermore, treatment with NO donors (topical application of nitroglycerine cream) has been shown to be very effective in prevention of post-menopausal bone loss (Wimalawansa, 2010). In another study, Marathe et al have shown that the osteoprotective effect of 17β-estradiol occurs through the NO/cGMP/PKG signaling module (Marathe, 2012). Based on these background studies in our laboratory, the NO/cGMP/PKG signaling pathway appears as one of the promising target for therapeutic intervention of bone loss. Studies focused on understanding the mechanism(s) and regulation of eNOS activation has been an area of intense investigation (Fleming, 2010). eNOS is regulated through Ca^{2+}/Calmodulin and phosphorylation. Mechanical stimuli such as fluid shear stress leads to eNOS phosphorylation and activation at Serine 1177 by PKA without increasing Ca^{2+} levels resulting in increased NO production(Fleming, 2010). Protein kinase Akt/PKB also activates eNOS by serine 1177 phosphorylation. The other phosphorylation sites that play an Important role in regulating eNOS activity include $pSer^{635}$, $pSer^{617}$, $pThr^{497}$, $pTyr^{657}$, and $pTyr^{81}$ (Fleming, 2010). However, under hyperglycemic conditions, the increased *O*-linked N-acetylglucosamine modification of

eNOS hinders Ser 1177 phosphorylation by Akt and thus lowers eNOS activity in endothelial cells (Du, 2001).

eNOS is a signaling complex that associates with proteins such as Calmodulin (CaM), Caveolin-1, and Hsp90. This association also functions as an important determinant in, regulating its activity. CaM binds with eNOS to facilitate the electron transfer necessary for NO synthesis from L-arginine. Caveolin-1 antagonizes CaM binding and thus decreases enzyme activity. Alternatively, Hsp90 association with eNOS increases NO production. However, Hsp90 has been found to be translocated to the cell surface in the aortic endothelium of diabetic mice as well as in vitro in hyperglycemic conditions, thereby impairing Hsp90 and eNOS interaction (Lei, 2006). Thus, decreased eNOS-Hsp90α interaction is one of the mechanisms contributing to reduce NO production in diabetic aortic endothelial cells. Based on these background studies, we hypothesize that reduced NO production leading to defective NO/cGMP/PKG II signaling contributes to decreased osteoblasts proliferation resulting in bone loss in diabetic patients.

The elevated ROS found in patients with DM oxidizes the heme iron on sGC, rendering it resistant to NO (Stasch, 2006). We hypothesize that treatment with cGMP elevating agents will circumvent the relative lack of NO in diabetic osteoblasts leading to increased osteoblast proliferation and survival. Cinaciguat (BAY 58-2667) directly activates sGC in a heme-independent manner and hence could be a potential treatment for osteoporosis in patients with DM.

The activating protein-1 (AP-1) transcription factor complex composed of fos and jun transcription factors have been identified to be important in bone development based

on gain and loss of function mutations of fos genes in mice. *c*-fos expression is involved in fracture healing and increases the expression of osteoblastic markers such as alkaline phosphatase and osteocalcin (Ohta, 1991). Fra-1 knockout mice show decreased bone matrix formation by osteoblasts and chondrocytes and show symptoms of osteopenia (Eferl, 2004). Transgenic mice overexpressing fra-1 have increased bone formation resulting from increased osteoblast differentiation and thus more mature osteoblasts (Jocum, 2000). Mice lacking fra-2 are osteopenic due to deficient osteoblasts and chondrocytes while overexpressing fra-2 increases osteoblast differentiation suggesting that fra-2 is involved in bone matrix formation (Bozec, 2010). FSS mediated activation of the NO/cGMP/PKG II signaling pathway leads to ERK and Akt mediated induction of fos family genes resulting in increased osteoblast proliferation (Rangaswami, 2009) . The anti-apoptotic effects of 17β-estradiol in murine POBs and MLO-Y4 osteocyte-like cells require NO/cGMP/PKG mediated activation of Erk and Akt (Marathe, 2012). Restoring ERK levels in ERK1/2 deficient neoplastic mouse JB6 cells reconstituted AP-1 activation, indicating that ERK1/2 is required for activation of AP-1 (Watts, 1998). Overexpression of Akt in mesangial cells increased AP-1 activation suggesting Akt is upstream and mediates AP-1 activation (Wu, 2009). Therefore, ERK and Akt mediates regulation of fos family gene expression necessary for osteoblast differentiation and bone formation.

Current therapies against osteoporosis such as bisphosphonates, estrogen agonists/antagonists, and hormone replacement therapy target osteoclast hyperactivity (Eriksen, 1999; Grasser, 1997; Khosla, 2012); however, osteoporosis in diabetic patients is due to impaired osteoblast function leading to decreased bone formation (Hamann,

2012). In addition, Salt et al. showed that high glucose inhibits insulin-stimulated NO production (Salt, 2003). Nitroglycerin can activate osteoblasts and has already been seen to be effective in treating osteoporosis in postmenopausal women, however continuous usage may lead to nitrate tolerance (Thadani, 1997). Similarly, parathyroid hormone treatment stimulates osteoblasts to form new bone; however, treatment is limited to 2 years because extended administration has been shown to lead to osteosarcoma in rats (Vahle, 2002). Based on the bone phenotype observed and considering the anabolic signaling of NO/cGMP/PKGII, we hypothesize that a defective NO/cGMP/PKGII signaling pathway is involved in deficient osteoblast proliferation in DM. In addition, cinaciguat may be a potential therapeutic drug to treating Type 1 diabetes-associated osteoporosis; therefore, we examined its effects on NO/cGMP/PKG II signaling and osteoblast proliferation.

MATERIALS AND METHODS

Materials

Antibodies against ERK1 Tyr $(P)^{202}$, Akt $Ser(P)^{473}$, VASP $Ser(P)^{239}$, Hsp90, RPTP-α and Total Akt were obtained from Cell Signaling. Antibodies against total Erk1, eNOS, α-tubulin, and β-actin were from Santa Cruz Biotechnology, Inc. Bromodeoxyuridine (BrDU), deoxyribonuclease I (DNase I), and anti-BrdU antibody was from Sigma. Antibodies against PKG I, and PKG II were from Abnova. The cyclic GMP agonist 8-pCPT-cGMP was from Biolog. The soluble guanylyl cyclase (sGC) activator cinaciguat (BAY 58-2667) was from AdipoGen. The NO donor 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA-NONOate) was from Cayman.

Cell Culture

Murine MC3T3-E1 (osteoblastic cells with high differentiation potential (clone 4, hereafter referred to as MC3T3 cells) were from American Tissue Culture Collection). MC3T3 cells were maintained in ascorbate-free-Minimal Essential Medium (MEM) supplemented with 10% FBS (Fetal Bovine Serum). Cells were grown in 100-mm-diameter dishes and passaged 1:6 twice a week.

To simulate the normoglycemic and hyperglycemic condition in MC3T3 cells, cells were maintained in media containing 1.0 (5 mM) and 4.5 g/l (25 mM) glucose respectively for 3 d prior to the experiment.

Generation of Type I diabetic mice and isolation of primary osteoblasts

In collaboration with Dr. Wolfgang Dillmann, mice with type I diabetes mellitus (DM I) were generated by intraperitoneal injection of streptozotocin (STZ

40 mg/kg) (Sigma) dissolved in 10 mM Na-citrate buffer (pH4.5) for 5 days. Hyperglycemia > 300 mg/dl was documented after 7 d. Primary osteoblasts (POBs) were isolated from tibial diaphyses four weeks later (at the age of 16 weeks). Diabetic POBs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, while POBs from control mice were maintained in 5 mM glucose. The media was supplemented with 10% FBS, penicillin streptomycin, and fungizone. Cells were used <six passages. The primary osteoblasts were characterized as described below.

Osteoblast Characterization--Alkaline Phosphatase Staining

Murine primary osteoblasts were cultured in DMEM supplemented with 10% FBS, ascorbic acid (100 μg/mL), β-glycerophosphate (5 mM) and dexamethasone (10 nM) for 2 weeks to induce differentiation and the alkaline phosphatase staining was performed as described previously (Promocell). Briefly, cells were fixed with 3.7% formaldehyde and then washed once with PBS. Cells were stained using the staining solution for 30 min. The reaction was stopped using the stop solution and photomicrographs were taken using Nikon microscope (10x magnification).

Staining Solution: 0.1 M Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.6 mM NBT, 0.6 mM 5-Bromo- 4-Chloro-3-Indolyl Phosphate (BCIP

Stop Solution: 10mM Tris HCl (pH 8.0) 1 mM EDTA

Vitamin D3 induced Osteocalcin expression

Differentiated murine primary osteoblasts were treated with vitamin D3 (10 nM) for 48h to induce osteocalcin expression. Quantitative RT-qPCR was used to quantify osteocalcin mRNA levels.

RNA Isolation, cDNA Synthesis and Quantitative RT-qPCR

RNA was extracted using TriReagent™ (Molecular Research Center, Inc.); cDNA was synthesized using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. *c-fos* was amplified with primers 5'-CGCAGAGCTCGGCAGAAGG-3' (sense) and 5'-TCTTGCAGGCAGGTCGGTGG-3' (anti-sense). *fra-1* was amplified with 5'-TGCGAGCAGATCAGCCCAGAG-3' (sense) and 5'-CTGGAGAAAGGGAGATGCAAGG-3' (anti-sense). *fra-2* was amplified with 5'-GGAGGAGAAGCGTGCAATCC-3'(sense) and 5'-GGGGCTGATTTTGCACACG-3'(anti-sense). *osteocalcin* was amplified with 5′-TGACGAGTTGGCTGACCACATC-3′ (sense) and 5′-GCAAGGGGAAGAGGAAAGAAGG-3′(antisense). *gapdh* was amplified with 5'-TGTCTTCACCACCATGGAGAACG -3' (sense)and 5'-GTGGATGCAGGGATGATGTTCTG-3' (anti-sense). PCR conditions were 30 s of denaturation at 95 ˚C, 45 s of annealing at 60 ˚C, and 1 s extension at 72 ˚C for 40 cycles. Quantitative RT-PCR was performed using an MX3000 real time PCR detection system (Stratagene) and IQ™ SYBR Green Supermix (Bio-Rad). Melting curves after 40 cycles confirmed a single PCR product for each primer pair. Relative changes in mRNA expression were analyzed using the $2^{-\Delta\Delta Ct}$ method, with *gapdh* serving as an internal reference to correct for differences in RNA extraction or reverse transcription efficiencies (Livak, 2001).

Bromodeoxyuridine Labeling

Murine primary osteoblasts were plated on glass coverslips. Cells were serum-starved, followed by treatment with cGMP or Cinaciguat (BAY 58-2667) for 1 h and labeled with 200 μM BrdU for 18 h. Cells were fixed and permeabilized with 3.7 %

formaldehyde and Triton X-100 respectively. The cells were incubated in DNase I(Sigma), blocked with 2 % BSA, stained with anti-BrdU antibody and Hoechst 33342. Cells were visualized with a Leica fluorescent microscope and analyzed as described (Rangaswami, 2010).

NO Quantitation

Murine primary osteoblasts (POBs) isolated from the control and STZ-induced type I diabetic mice were subjected to low or high glucose conditions for 48 h before incubation in phenol red-free DMEM for 24 h. The media were collected and nitric oxide production was quantified with a two-step colometric assay kit measuring nitrite and nitrate accumulation according to the manufacturer's protocol (Active Motif).

eNOS Immunoprecipitation

MC3T3 cells were seeded on 15 cm plates and treated with low or high glucose media for 48 h. Cells were washed twice with cold PBS, and then lysed with LB (0.5 % Triton X-100, 50 mM Tris HC pH 7.4, 0.15 M NaCl, 0.1 mM EDTA supplemented with protease and phosphatase inhibitors). Cells were harvested and subjected to homogenization with an insulin syringe. Samples were centrifuged at 13,200 rpm for 10 min at 4 ˚C. A tenth of the supernatant was saved for input control while the remaining lysate was used for immunoprecipitation using anti-eNOS antibody(1 µg) for 3 h. Control IgG was used during immunoprecipitation as a negative control. Following which, protein G-Agarose beads (Calbiochem) were added to samples and rotated overnight. The beads were washed first with a high salt wash buffer (500 mM NaCl, 100 mM Tris HCl pH 7.4, 0.5% NP-40) and twice with binding buffer (10 mM Tris HCl pH 7.9, 2 mM $MgCl_2$, 0.15 mM NaCl, 10% glycerol). Hot SDS sample buffer was used for

elution and proteins were separated by 15% SDS-PAGE and analyzed by western blotting using anti-Hsp 90 antibody. The immunoprecipitates were also analyzed using anti-eNOS antibody.

Cell Fractionation

MC3T3 cells were fractionated as described[3]. To reiterate, cells were lysed (10 mM Hepes pH 7.5, 1 mM EDTA, .5 mM EGTA, 1 mM $MgCl_2$, 1mM DTT, and protease inhibitors) and extracted by Dounce homogenization. After centrifuging at 1000 rpm at 4˚C for 5 min, the nuclear pellet was discarded and the supernatant was centrifuged at 37,000 x g for 30 min. The supernatant was saved as the cytosolic fraction while the pellet was resuspended in lysis buffer (1% Triton X-100, 500 mM NaCl, 60 mM β-octyl glucopyranoside) and saved as the membrane fraction. Cytosolic fractions were concentrated via TCA precipitation as described (Sanchez, 2001). The cytosolic and membrane fractions were separated by SDS-PAGE and analyzed by western blot using PKG I and PKG II specific antibodies.

Western Blot Analysis

The protein samples were separated by 9% SDS-PAGE unless specified otherwise. After transfer, PVDF membranes were blocked with 5% milk in Tris buffered saline containing 0.1% Tween 20 (TBST) and incubated with primary antibody followed by Horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescense (Thermo) were used to generate western blots as described previously (Guidi, 1997). Films were analyzed using ImageJ software (nih.gov).

Statistical Analysis

Data were analyzed by one-way ANOVA with Bonferroni multiple comparisons analysis against the control group using GraphPad Prism. A p value <0.05 was considered statistically significant. Bar graphs represent mean ± SEM of three independent experiments unless stated otherwise.

Materials and Methods, in part is currently being prepared for submission for publication of the material. Pilz, Renate B; Kalyanaraman, Hema; Chan, Geraldine C; Scott, Brian T; Dillman, Wolfgang. The dissertation author was the primary investigator and author of this material.

RESULTS

Osteoblast Characterization of osteoblasts by Alkaline phosphatase (ALP) staining and Vitamin D3 induced osteocalcin expression

Primary osteoblasts isolated from the control and streptozotocin induced type I diabetic mice were used to in various experiments in this project. To confirm that cells isolated from control and diabetic mouse tibiae were osteoblasts, cells were cultured in differentiation media for 14 d and used for alkaline phosphatase staining. In Fig. 1B, the osteoblasts cultured in differentiation media were treated with vitamin D3 for 48 h and osteocalcin mRNA level was analyzed by qPCR. After 2 weeks in culture, ~80% of both control and diabetic mPOB stained positive for ALP (Fig 1A) suggesting that mPOB used in experiments were indeed osteoblasts. Control and diabetic mPOB treated with vitamin D3 for 2 days also showed a 4-fold increase in osteocalcin mRNA levels (Fig 1B).

Diabetic Osteoblasts have decreased NO production and reduced eNOS-Hsp 90α association

The NO/cGMP/PKG signaling pathway is involved in osteoblast maturation, proliferation, and survival (Marathe, 2012; Rangaswami, 2012). Previous reports indicate that treatment with NO donors (Nitroglycerine) increases osteoblast activity as shown by increased bone mineral density (BMD) in rats with ovarectomy induced osteoporosis (Wimalawansa, 1996). . To determine if NO production is altered in diabetic osteoblasts, media from control and diabetic mPOB were collected after 24h and NO levels were measured. We found that diabetic mPOB had significantly reduced basal NO production compared to control mPOB (Fig 2A). In order to analyze whether the reduced NO

production observed in diabetic osteoblasts is due to altered eNOS-Hsp90α interaction, immunoprecipitates of endogenous eNOS from control and diabetic mPOB were analyzed on a western blot using anti-Hsp90α antibody. We found that eNOS-Hsp 90 binding was nearly abolished in diabetic mPOB (Fig 2B) suggesting that eNOS activity might be diminished in diabetic osteoblasts, resulting in decreased NO production.

Diabetic mPOB have decreased PKG II protein expression

PKG I and II mediates the anti-apoptotic functions of 17-β-estradiol resulting in increased osteocyte survival (Marathe, 2012) Further, mechanical stimulation of osteoblasts increases osteoblast proliferation through the NO/cGMP/PKG II pathway (Rangaswami, 2009). To determine if diabetic osteoblasts have altered PKG levels, cytosolic and membrane fractions were prepared from the control and diabetic osteoblasts and PKG I and II protein levels were analyzed by western blot using their respective antibodies. Our data indicated that PKG I expression between control and diabetic groups remained unchanged; however, PKG II expression in diabetic mPOB was reduced by over 40% compared to control mPOB (Fig 3).

Reduced sensitivity to NO signaling in diabetic osteoblasts

To examine the effect of hyperglycemia on NO signaling in osteoblasts, MC3T3 cells were cultured in normoglycemic (5 mM glucose) and hyperglycemic (25 mM glucose) conditions followed by treatment with increasing concentrations of the NO-donor PAPA-NONOate. While 0.1 μM PAPA-NONOate was sufficient to increase Akt and ERK activation by over 2-fold in normoglycemic MC3T3 cells, hyperglycemic MC3T3 cells required 100-fold more concentrated PAPA-NONOate treatment for comparable Akt and ERK activation (Fig 4). This suggests that diabetic osteoblasts have

reduced sensitivity to the NO donor PAPA-NONOate and hyperglycemia impedes NO signaling.

Mannitol, as an osmolarity control, confirmed that osmotic pressure from hyperglycemia does not lead to defective Akt and Erk activation. cGMP enhanced Erk activation in MC3T3 cells cultured in media with mannitol to the same extent as normoglycemic conditions while hyperglycemic MC3T3 cells had lower Akt and Erk activation (Fig 5).

Cinaciguat induces VASP phosphorylation and enhances fos family gene expression

Cinaciguat (BAY 68-2667) (Fig 6A) activates heme-oxidized sGC to produce cGMP resulting in PKG activation. VASP phosphorylation at Ser^{259} confirmed sGC activity in human primary osteoblasts (hPOB) treated with cinaciguat. Treatment of hPOBs with increasing concentrations of cinaciguat (0.01μM, 0.1 μM and 1 μM) resulted in a dose responsive increase in VASP phosphorylation (Fig 6B, C).

To determine the effect of cinaciguat (BAY 68-2667) and cGMP on *fos* family gene expression, MC3T3 cells were treated with 100 nM BAY 68-2667 or 100 μM cGMP for 2h. qPCR data showed that both cinaciguat and cGMP effectively increased *fos*, *fra 1* and *fra 2* expression in these cells (Fig 6)

ERK and Akt activation effectively induced by cGMP and cinaciguat in control and diabetic osteoblasts

To analyze the effect of hyperglycemia on cGMP and cinaciguat induced ERK and Akt activation in primary cells, control and diabetic mPOB were treated with 100 μM cGMP or 100 nM cinaciguat. Our western blot data showed that both cGMP and cinaciguat induced Akt activation by 4-fold in both control and diabetic mPOB but the

effect was less pronounced in diabetic mPOB. However, ERK activation was similar between control and diabetic osteoblasts with induction up to 2.5 fold. (Fig 7)

Cinaciguat induces osteoblast proliferation

Previous studies have shown that cGMP exerts a pro-proliferative effect in osteoblasts (Rangaswami, 2010). To determine if cinaciguat (BAY 68-2667) as an NO and heme independent sGC activator generates the same effect, mPOB were treated with cGMP or cinaciguat and BrDu incorporation was examined. Our data showed that cGMP and cinaciguat stimulated BrDu incorporation by a factor of 3 and 5 respectively (Fig 8) suggesting that BAY 68-2667 is as effective if not more effective than cGMP in elevating osteoblast proliferation.

DISCUSSION

Osteoporosis is an under-studied complication of diabetes mellitus. Diabetes Mellitus Type 1-associated osteoporosis leads to increased fracture risk and delayed fracture healing (Espallargues, 2001). Studies in diabetic rodent models have shown decreased bone mineral density and mineral apposition rate within 4-6 weeks after confirming hyperglycemia (McCabe, 2007). The anabolic role of NO/cGMP/PKG II signaling has been shown to be important in osteoblast proliferation and survival. Mechanotransduction mediated activation of the NO/cGMP/PKG II signaling cascade results in increased ERK and Akt mediated fos family genes leading to osteoblast proliferation (Rangaswami, 2009). Further, 17β-estradiol increases osteocyte survival dependent on NO/cGMP/PKG mediated activation of ERK and Akt (Marathe, 2012). However, our work suggests that this pathway might be impaired by diabetes and this may contribute to the diabetic bone phenotype. Diabetic osteoblasts were less sensitive to NO signaling and cGMP induced ERK and Akt phosphorylation. This may result from impaired NO activation of sGC or reduced PKG II protein levels. However, cinaciguat stimulated Akt and ERK phosphorylation in diabetic osteoblasts. Cinaciguat also induced fos family gene expression, and osteoblast proliferation.

Decreased levels of eNOS-Hsp90α association observed in diabetic osteoblasts suggests that eNOS is less active in diabetes than in control osteoblasts. Previous research has shown that high glucose and diabetes increases Hsp90α translocation to the cell surface and prevents Hsp90α and eNOS interaction and thus decreases eNOS activation (Lei, 2006). Since eNOS is the main source of NO in osteoblasts, decreased eNOS activity would significantly decrease NO levels in osteoblasts. This would explain the

resultant lower levels of NO observed in diabetic osteoblasts. NO is essential for it stimulates osteoblasts proliferation, differentiation, and survival.

Under oxidative stress, sGC heme groups can become oxidized which would interfere with heme-dependent NO activation (Munzel, 2005). Erk and Akt phosphorylation was nearly undetectable with low concentrations of NO-donor PAPA-NONOate treatment and only concentrations as high as 100-fold restored Erk and Akt activity in diabetic osteoblasts. This confirms NO activation is indeed impaired in diabetic osteoblasts and indirectly suggests sGC is oxidized. Therefore, a new method to activate sGC is necessary. Cinaciguat activates sGC in a NO- and heme- independent manner and should be able to activate sGC even under oxidative stress. To measure sGC activity, PKG induced VASP phosphorylation was examined. Treatment with cinaciguat induced VASP phosphorylation in a dose dependent manner, confirming that cinaciguat activates sGC.

Cinaciguat effectively increased fos family mRNA to the same extent as cGMP. Since cinaciguat stimulates cGMP production, their respective effects on fos family gene expression were expected to be very similar. Fos family overexpression has been shown to increase osteoblast differentiation and bone formation; thus targeting fos family mRNA overexpression may have bone restorative effects in osteoporosis.

Similarly, cinaciguat was able to stimulate Erk and Akt phosphorylation. Akt activation by cGMP or cinaciguat in diabetic osteoblasts was decreased compared to control; however, this might have resulted from lower PKG II protein levels. PKG II has been shown in our lab to mediate cGMP-induced osteoblast proliferation via activation of

Akt and Erk (Rangaswami, 2009). Decreased PKG II expression handicaps cGMP induced Akt phosphorylation.

Despite comparatively lower levels cGMP and cinaciguat induced Erk and Akt phosphorylation, treatment with cinaciguat was able to increase fos family gene expression and osteoblast proliferation. This suggests that treatment with cGMP elevating agents such as cinaciguat could be a novel and effective therapeutic strategy to treat diabetes-associated osteoporosis. Therefore, the next step in this project is to test whether cinaciguat increases bone formation and BMD *in vivo.*

A study composed of three groups—1) control mice, 2) Streptozotocin (STZ)-induced Type I diabetic mice, and 3) diabetic mice treated with cinaciguat—can be devised to study effect of cinaciguat treatment in diabetes induced osteoporosis *in vivo.* We can use micro-CT imaging and histomorphometry to examine the static and dynamic parameters in diabetic bone with or without treatment. Micro-CT imaging analysis would elucidate differences in bone density while histomorphometry examines differences in mineral apposition rates and bone formation rates. RNA can also be isolated from mice tibiae to measure differences in gene expression such as osteocalcin and PKG among other genes.

It is also important to further understand which components of the NO/cGMP/PKG II pathway are impaired due to diabetes. Although it appears that deficient PKG II levels might result in decreased Erk and Akt activation by both NO-donors and cGMP, it does not alone explain the severity at which NO-induced Erk and Akt phosphorylation is impaired. It is necessary to further understand sGC activity with or without cinaciguat induction by measuring the levels of cGMP that is produced in both

diabetic and control osteoblasts. This would inform us what levels of cinaciguat is sufficient to restore proper cGMP levels in diabetic osteoblasts. Also, although it has been shown in aortic cells that sGC is oxidized in diabetes, it has yet to be shown in osteoblasts; therefore, more experiments should be done to verify whether or not NO activation of sGC is indeed impaired due to sGC oxidization.

Overall, we have been able to show that multiple steps of the NO/cGMP/PKG II signaling pathway is defective in diabetic osteoblasts. In diabetic osteoblasts, eNOS activation to produce NO is impaired, sGC sensitivity to NO signaling is reduced, and PKG II protein levels were markedly decreased. However, cinaciguat is a NO- and heme independent cGMP-elevating agent that is a potential therapeutic agent in increasing bone formation and thus treat osteoporosis in diabetic patients.

FIGURES

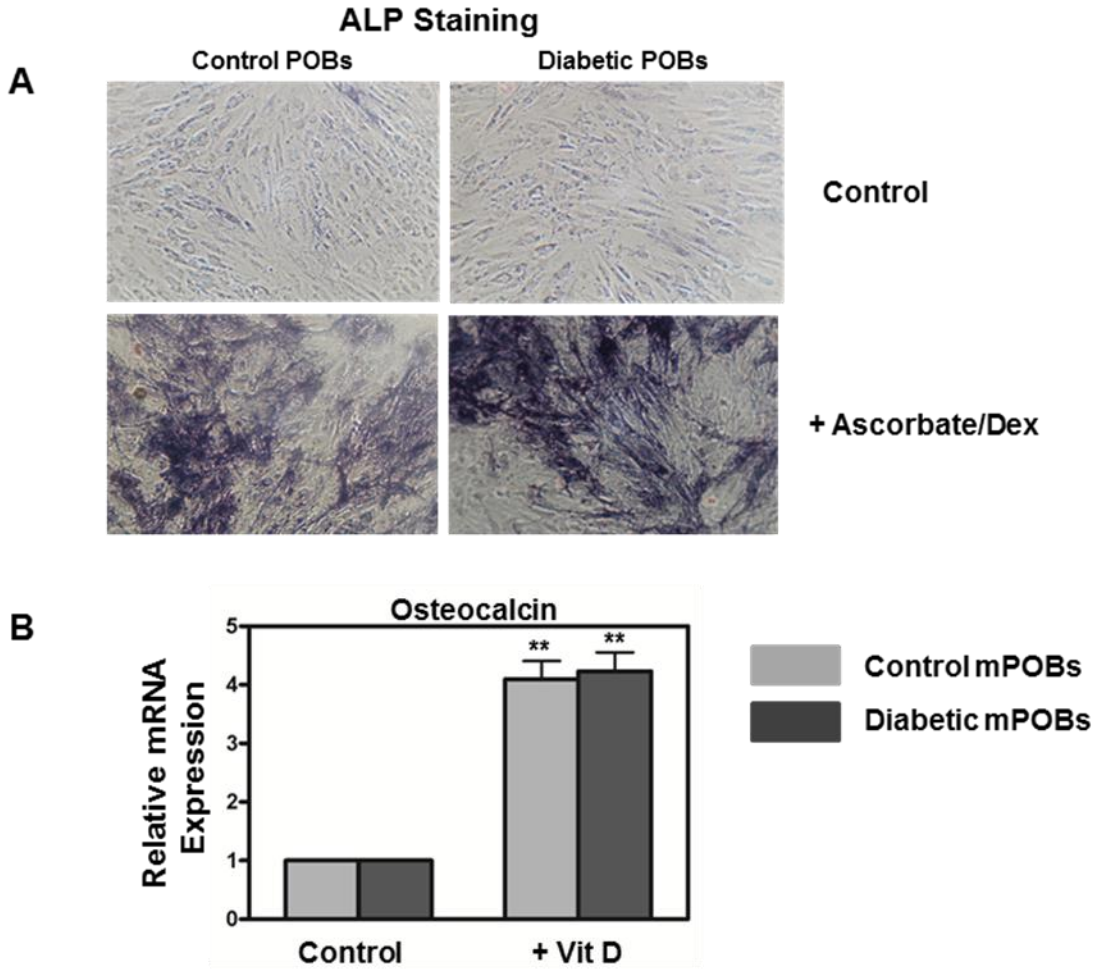


Figure 1: Characterization of Murine Primary Osteoblasts. *A,* Murine primary osteoblasts isolated from the control and type I diabetic mice were cultured for 14 d in absence (control) or presence of100 mM ascorbate and 10 nM dexamethasome (A/D) to induce differentiation. Alkaline phosphatase activity was assessed by cytochemical staining (A) using 5-Br-4-Cl-3-indolyl phosphate and nitro blue tetrazolium chloride. *B,* Differentiated mPOBs were treated with vehicle (control) or 1,25 dihydroxy vitamin D3 (Vit D, 1 nM) for 24 h prior to measuring osteocalcin mRNA levels by real time RT-PCR and normalized relative to gapdh mRNA levels as described under "Materials and Methods." (** $p < 0.01$, n=3). The relative mRNA levels found in undifferentiated mPOB were assigned a value of 1.

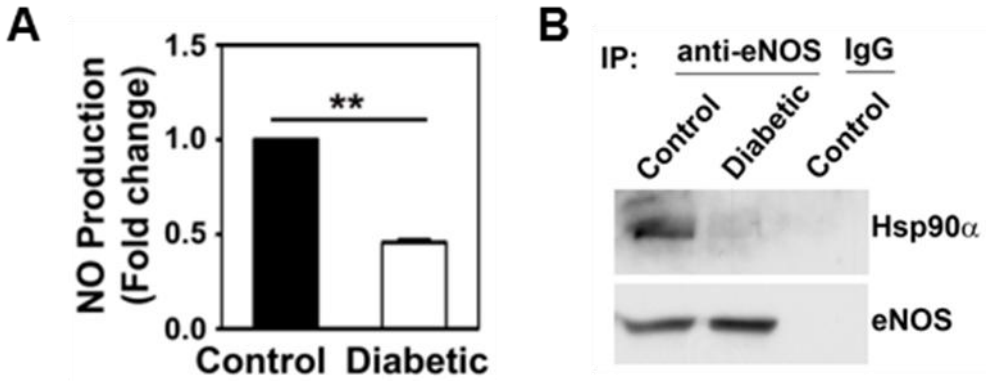


Figure 2: Decreased NO production and reduced eNOS-Hsp90α association in diabetic osteoblasts. *A,* Control and diabetic mPOB were cultured in phenol free DMEM and the basal nitrate and nitrite (NOx) concentrations were measured as described in Methods. (** $p<0.01$, n=3). *B,* Cell lysates prepared from the control and diabetic osteoblasts were subjected to immunoprecipitation with anti-eNOS antibody or control IgG antibody and analyzed by western blot using antibodies specific for Hsp 90a and eNOS.

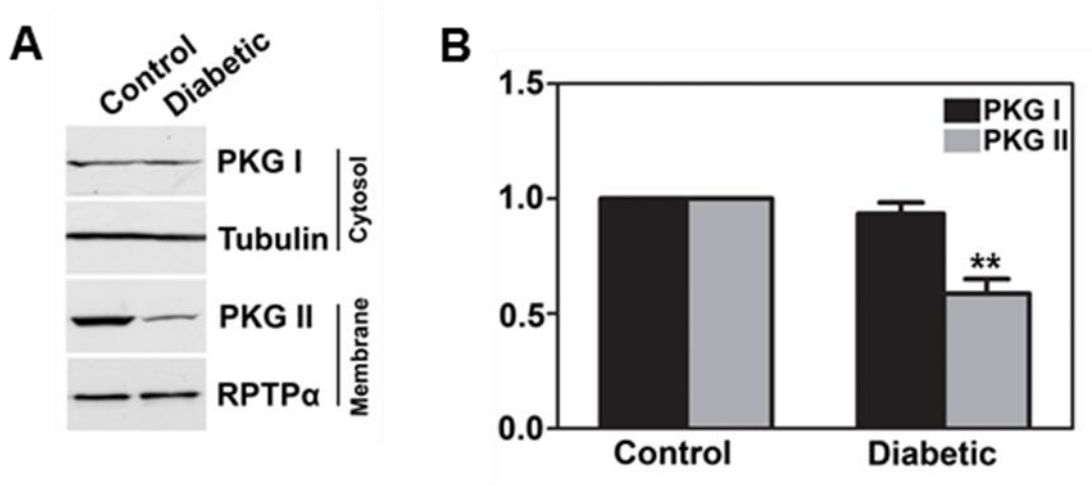


Figure 3: Decreased PKG II expression in diabetic osteoblasts. *A,* Control and diabetic mPOB were extracted by Dounce homogenization and fractionated by differential centrifugation. Cytosolic and membrane fractions were analyzed by western blot using antibodies specific for PKG I, α-tubulin, PKG II, and RPTP-α. *B,* Densitometric analysis of the western blots from three independent experiments was performed using the Image J software. The intensity of the bands found in control mPOB were assigned a value of 1. **, $p<0.01$ for comparison between control and diabetic mPOB for PKG II.

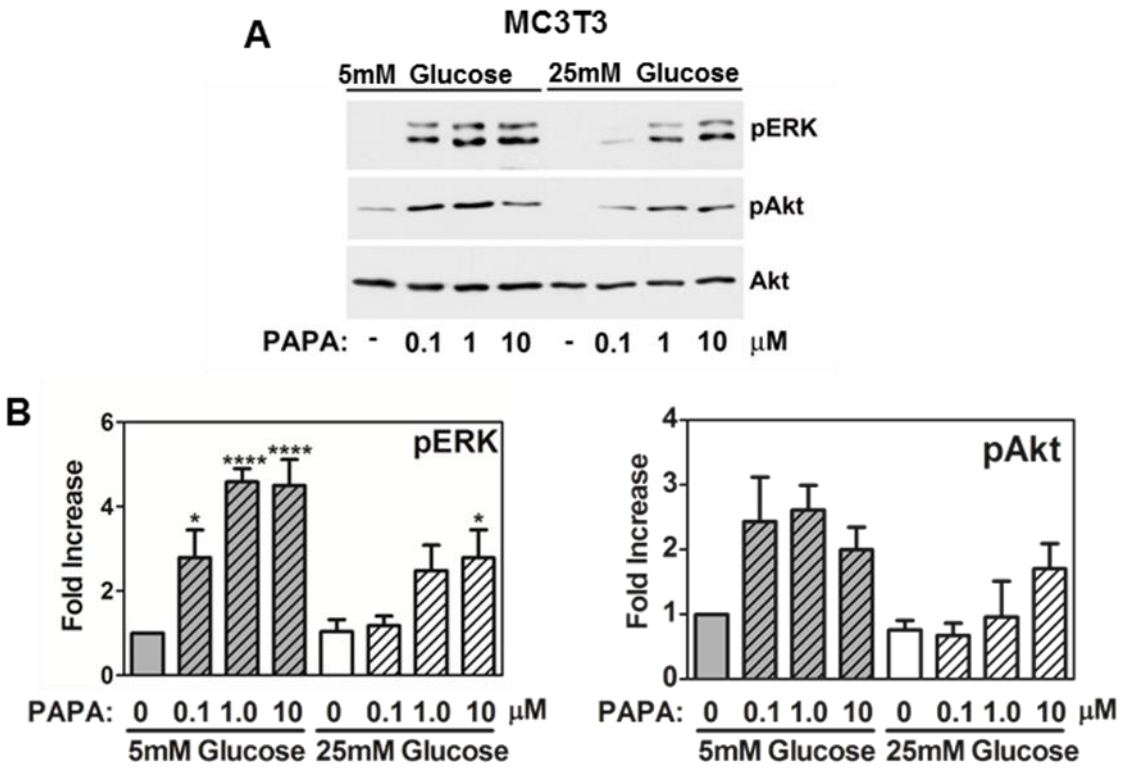


Figure 4: Hyperglycemia impedes Akt and ERK activation by NO. *A,* serum-starved MC3T3 cells cultured in normoglycemic *(lane 1-4)* and hyperglycemic *(lane 5-8)* conditions were treated with PAPA-NONOate *(lanes 2-4 and 6-8)* at the indicated concentrations for 5 minutes. Cell lysates were analyzed by western blot using antibodies specific for Akt pSer^{473}, ERK1 pTyr^{202}, or β-actin. *B,* Western bots from four independent experiments were analyzed by densitometry and the intensity of the band found in normoglycemic untreated cells was assigned a value of 1.

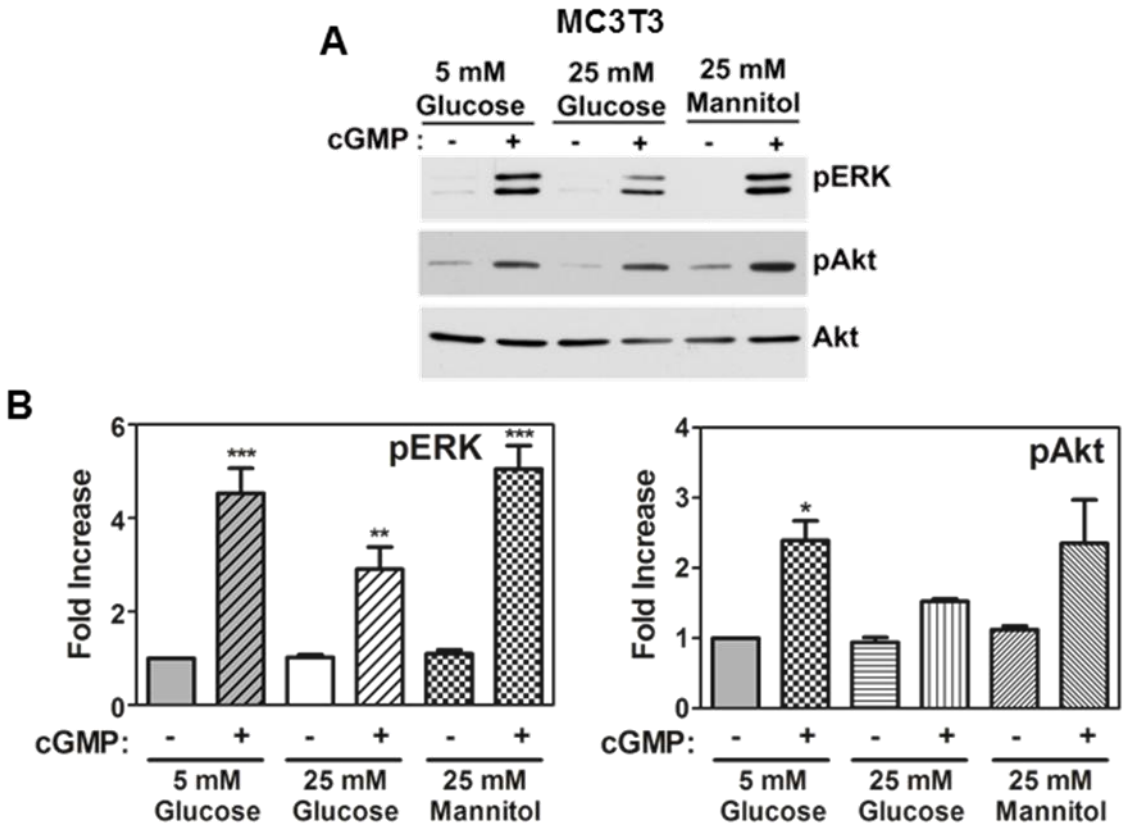


Figure 5. Defective ERK/Akt signaling in diabetic osteoblasts. *A,* MC3T3 cells were cultured in MEM containing 5 mM(lanes 1 and 2) or 25 mM glucose (lanes 3 and 4) or 25 mM mannitol (lanes 5 and 6). Serum-starved cells were treated with 100 µM 8-cPT-cGMP for 10 minutes. Cell lysates were analyzed by western blot as described in Fig 4A. *B,* Western blots were analyzed by densitometry and the intensity of the band found in normoglycemic untreated cells were assigned a value of 1. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ for comparison between untreated and treated cells per culture condition, n=3.

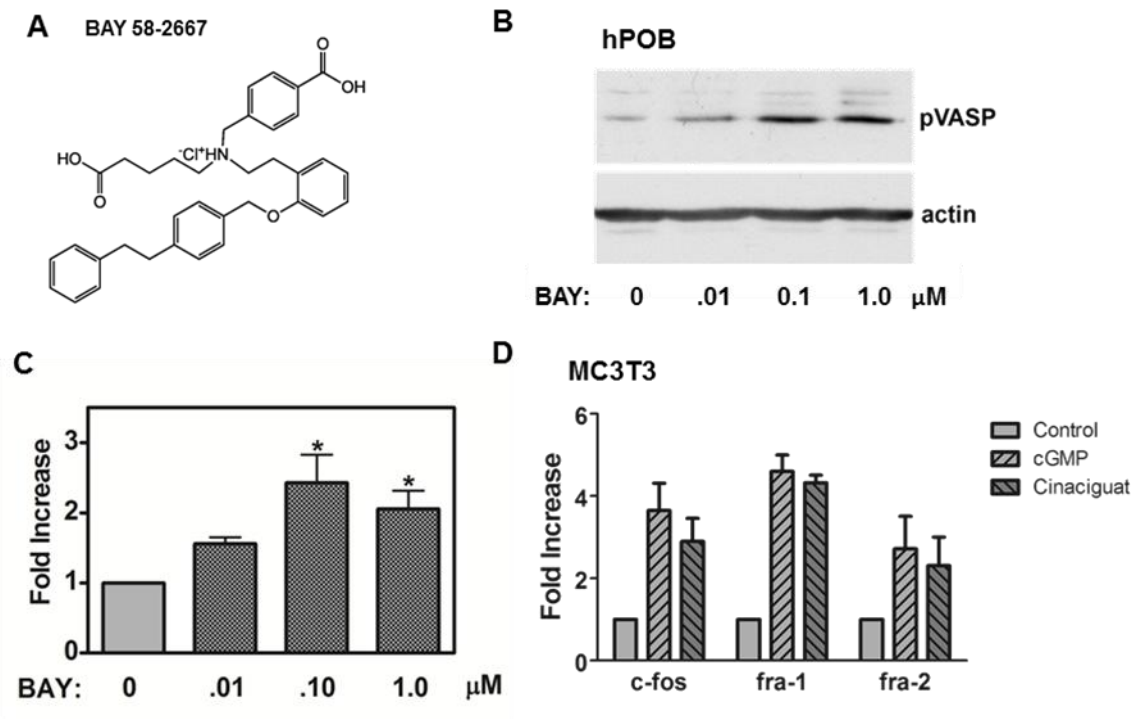


Figure 6: Effect of Cinaciguat on VASP activation and Fos family gene expression. *A*, chemical structure of Cinaciguat (BAY 58-2667). *B*, serum-deprived hPOB were treated with BAY 58-2667 at the indicated concentrations for 10 min. Cell lysates were analyzed by western blot using antibodies specific for VASP phosphorylation at Ser^{239} or β-actin. *C*, western bots were analyzed by densitometry and the intensity of the band found in untreated cells was assigned a value of 1. *, $p<0.05$ for comparison between control and treated cells. n=3 *D*, Serum-deprived MC3T3 cells cultured in hyperglycemic conditions were treated with 100 µM 8-pCT-cGMP or 100 nM BAY 58-2667 for 1 hr and *c-fos*, *fra-1*, and *fra-2* mRNA levels were quantified by real time RT-PCR and normalized relative to *gapdh* mRNA levels as described under "Materials and Methods." *, $p<0.05$ for comparison between control and treated cells for normoglycemic and hyperglycemic conditions independently.

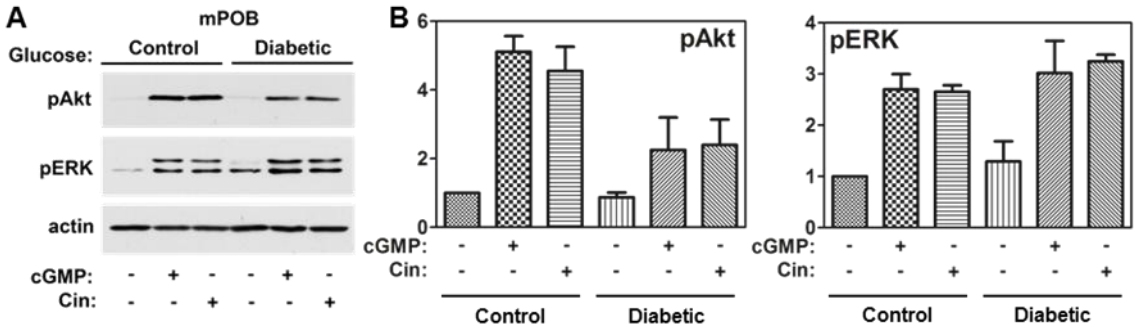


Figure 7: cGMP and Cinaciguat effectively induces ERK and Akt phosphorylation in control and diabetic osteoblasts. *A*, primary osteoblasts isolated from the control *(lanes 1-3)* and diabetic mice, *(lanes 4-6)* were serum-deprived prior to treatment with 100 µM cGMP *(lane 2 and 5)* or 100 nM BAY *(lane 3 and 6)* for 10 min. Cell lysates were analyzed by western blot as described in Fig 4A. *B*, western bots from two independent experiments were analyzed by densitometry and the intensity of the band found in normoglycemic untreated cells was assigned a value of 1.

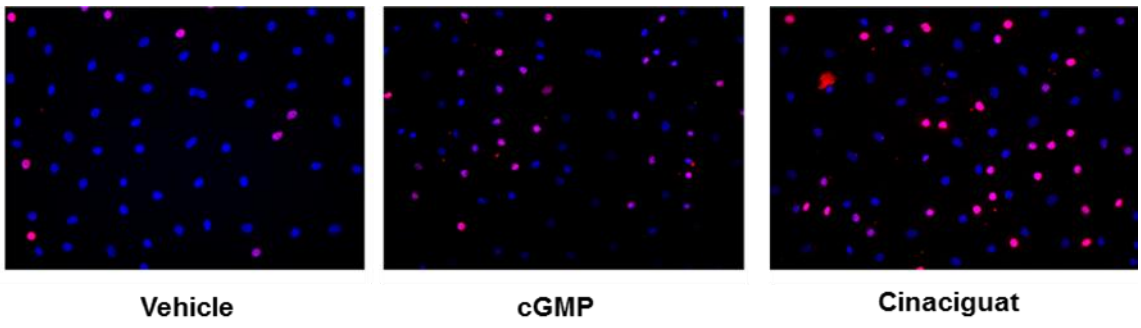


Figure 8: Cinaciguat increases osteoblast proliferation. Serum-starved mPOB were treated with 100 µM cGMP or 100 nM BAY 58-2667 for 1hr and labeled with 200 µM BrDU for 18 hr. BrDu incorporation into DNA was detected by immunofluorescence.

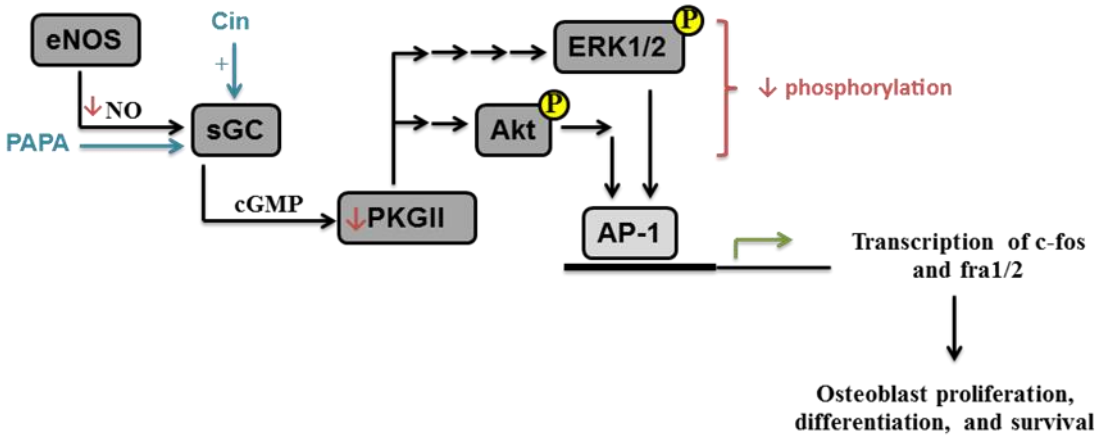


Figure 9: Defective NO/cGMP/PKG II Signaling in Diabetic Osteoblasts

Figures, in part is currently being prepared for submission for publication of the material. Pilz, Renate B; Kalyanaraman, Hema; Chan, Geraldine C; Scott, Brian T; Dillman, Wolfgang. The dissertation author was the primary investigator and author of this material.

REFERENCES

Armour KE, Armour KJ, Gallagher ME, Gödecke A, Helfrich MH, Reid DM, Ralston SH. (2001) Defective bone formation and anabolic response to exogenous estrogen in mice with targeted disruption of endothelial nitric oxide synthase. Endocrinology. 142(2):760-766

Blakytny R, Spraul M, Jude EB. (2011) The Diabetic Bone: A Cellular and Molecular Perspective. The International Journal of Lower Extremity Wounds. 10:16-32

Botolin S, McCabe LR. (2007) Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice. Endocrinology. 148(1):198-205

Bozec A, Bakiri L, Jimenez M, Schinke T, Amling M, Wagner E. (2010) Fra-2/AP-1 controls bone formation by regulating osteoblast differentiation and collagen production. J. Cell Biol. 190(6):1093:1106

Brandi ML, Hukkanen M, Umeda T, Moradi-Bidhendi N, Bianchi S, Gross SS, Polak JM, MacIntyre I. (1995) Bidirectional regulation of osteoclast function by nitric oxide synthase isoforms. Proc Natl Acad Sci U.S.A. 92(7):2954-8

Du X, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. (2001) Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. J. Clin. Invest. 108:1341-8

Eferl R, Hoebertz A, Schilling A.F, Rath M, Karreth F, Kenner L, Amling M, Wagner E. (2004) The Fos-related antigen Fra-1 is an activator of bone matrix formation. The EMBO Journal. 23:2789-99

Eriksen E, Langdahl B, Vesterby A, Rungby J, Kassem M. (1999) Hormone replacement therapy prevents osteoclastic hyperactivity: A histomorphometric study in early postmenopausal women. J Bone Miner Res.14(7)1217-21

Espallargues M, Sampietro-Colom, L, Estrada, M. D, Sola, M, de Rio L, Setoain J, Granados A. (2001) Osteoporosis International. Identifying Bone-Mass Related Risk Factors for Fracture to Guide Bone Densitometry Measurements: A Systematic Review of the Literature. Osteoporosis Int. 12:811-22

Fleming I. (2010) Molecular mechanisms underlying the activation of eNOS. Eur J Physiol. 459:793-806

Franke S, Rüster C, Pester J, Hofmann G, Oelzner P, Wolf G. (2011) Advanced glycation end products affect growth and function of osteoblasts. Clin Exp Rheumatol. 29(4):650-60

Grasser W, Pan L, Thompson D, Paralkar W. (1997) Common mechanism for the estrogen agonsist and antagonist activities of droloxifene. J. Cell Biol. 65(2):159-71

Gudi, T, Lohmann, S.M, Pilz R.B. (1997) Mol. Cell. Biol. 17, 5244-5254

Hamada Y, Fujii H, Fukagawa M. (2009) Role of oxidative stress in diabetic bone disorder. Bone. 45: S35-38

Hamann C, Kirshner S, Günther K, Hofbauer L. (2012) Bone, sweet bone—osteoporotic fractures in diabetes mellitus. Nat. Rev. Endocrinol. 8, 297-305

Jocum W, David J.P, Elliott C, Wutz A, Plenk H, Matsuo K, Wagner E. (2000) Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. Nat. Med. 6:980-4

Khosla S, Bilezikian J, Dempster D, Lewiecki M, Miller P, Neer R, Recker R, Shane E, Shoback, Potts J. (2012) Benefits and Risks of Bisphosphonate Therapy for Osteoporosis. J. Clin. Endocrinol Metab.97(7)2272-82

Lei H, Venkatakrishnan A, Yu S, Kazlauskas A. (2006) Protein Kinase A-dependent Translocation of Hsp90α Impairs Endothelial Nitric-oxide Synthase Activity in High Glucose and Diabetes. J. Biol. Chem. 282(13)9364-71

Livak K, Schmittgen T. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$. Methods. 25:402-8

Marathe N, Rangaswami H, Zhuang S, Boss G, Pilz R. (2012) Pro-survival effects of 17β-Estradiol on Osteocytes Are Mediated by Nitric Oxide/cGMP via Differential Actions of cGMP-dependent Protein Kinases I and II. J. Biol. Chem. 287(2)978-88

McCabe LR. (2007) Understanding the Pathology and Mechanisms of Type I Diabetic Bone Loss. Journal of Cell Biol. 102:1343-1357

McPherson H, Noble BS, Ralston SH. (1999) Expression and functional role of nitric oxide synthase isoforms in human osteoblast-like cells. Bone (NY). 24:179-85

Munzel T, Daiber A, Ullrich V, Mulsch A (2005) Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. Arterioscler Thromb Vasc Biol. 25:1551-7

Ohta S, Yamamuro T, Okumura H, Kasai R, Hiraki Y, Ikeda T, Kikuchi H, Konishi J. et al. (1991) Fracture healing induces expression of the proto-oncogene c-fos in vivo. Possible involvement of Fos protein in osteoblastic differentiation. FEBS Lett. 284(1)42-5

Promocell. Osteoblast Differentiation and Mineralization. PromoCell.

Rangaswami H, Marathe N, Zhuang S, Chen Y, Ye JC, Frangos JA, Boss GR, Pilz RB (2009) Type II cGMP-dependent Protein Kinase Mediates Osteoblast Mechanotransduction. J Biol Chem. 284:14796-14808

Rangaswami H, Schwappacher R, Marathe N, Zhuang S, Casteel D. E, Haas B, Chen Y, Pfeifer A, Kato H, Shattil S, Boss G. R, Pilz R. B. (2010) Cyclic GMP and protein kinase G control a Src-containing mechanosome in osteoblasts. Sci.Signal. 3, ra91

Rangaswami H, Schwappacher R, Tran T, Chan GC, Zhuang S, Boss GR, Pilz RB (2012) Protein Kinase G and Focal Adhesion Kinase Converge on a Src/Akt/β-Catenin Signaling Module in Osteoblast Mechanotransduction. J Biol Chem. 267(25):21509-19

Riggs BL, Wahner HW, Seeman E, Offord KP, Dunn WL, Mazess RB, Johnson KA, Melton LJ 3rd. (1982) Changes in bone mineral density of the proximal femur and spine with aging. Differences between the postmenopausal and senile osteoporosis syndromes. J Clin Invest. 70(4):716-23

Sanchez L. (2001) TCA Protein Precipitation. Protocols on line.
Schwartz AV, Sellmeyer DE (2004) Women, type 2 diabetes, and fracture risk. Curr Diab Rep. 4(5):364-9

Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, H S AK, Meurer S, Deile M, Taye A, Knorr A, Lapp H, Müller H, Turgay Y, Rothkegel C, Tersteegen A, Kemp-Harper B, Müller-Esterl W, Schmidt HH. (2006) Targeting the heme-oxidized nitric oxide receptor for selective vasodilation of diseased blood vessels. J Clin Invest.116(9):2552-61

Thadani U (1997) Nitrate tolerance, rebound, and their clinical relevance in stable angina pectoris, unstable angina, and heart failure. Cardiovasc Drugs Ther. 10:734-42

Waud CE, Marks SC Jr, Lew R, Bara DT (1994) Bone mineral density in the femur and lumbar vertebrae decreases after twelve weeks of diabetes in spontaneously diabetic-prone BB/Worcester rats. Calcif Tissue Int. 54(3):237-40

Vahle JL, Sato M, Long GG, Young JK, Francis PC, Engelhardt JA, Westmore MS, Linda Y, Nold JB (2002) Skeletal changes in rats given daily subcutaneous injections of recombinant human parathyroid hormone (1-34) for 2 years and relevance to human safety. Toxicol Pathol. 30(3):312-21

Verhaeghe J, Van Herck E, van Bree R, Moermans K, Bouillon R (1997) Decreased osteoblast activity in spontaneously diabetic rats. In vivo studies on the pathogenesis. Endocrine. 7(2):165-75

Watts RG, Huang C, Young MR, Li JJ, Dong Z, Pennie WD, Colburn NH (1998) Expression of dominant negative Erk2 inhibits AP-1 transactivation of neoplastic transformation. Oncogene. 17(26):3493-8

Wimalawansa SJ. (2000) Restoration of ovariectomy-induced osteopenia by nitroglycerin. Calcif Tissue Int. 66(1):56-60

Wimalawansa SJ. (2010) Nitric Oxide and Bone. Ann. N.Y. Acad. Sci.1192:391-403

Wu D, Peng F, Zhang B, Ingram AJ, Kelly DJ, Gilbert RE, Gao B, Krepinsky JC (2009) PKC-β1 mediates glucose-induced Akt activation and TGF-β1 upregulation in mesangial cells