This is to certify that the

dissertation entitled

Elevated Extracellular Glucose Elicits An Osmotic Response In Osteoblasts Involving Signaling And Transcription Factor Activation

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

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Date 8-10-2001

ELEVATED EXTRACELLULAR GLUCOSE ELICITS AN OSMOTIC RESPONSE IN OSTEOBLASTS INVOLVING SIGNALING AND TRANSCRIPTION FACTOR ACTIVATION

By

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AN ABSTRACT OF A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

2001

Professor Laura R. McCabe

ABSTRACT

ELEVATED EXTRACELLULAR GLUCOSE ELICITS AN OSMOTIC RESPONSE IN OSTEOBLASTS INVOLVING SIGNALING AND TRANSCRIPTION FACTOR ACTIVATION

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Investigation of osmotic stress in mammalian tissues has focused principally on kidney and the cellular responses to hyperosmolar conditions of 300 mOsm above normal. Recently, many studies have focused on physiologically relevant extracellular hyperosmolarity to increase our understanding of cellular osmotic response in diseases such as uremia, hypernatremia and diabetes mellitus. Insulin dependent diabetes mellitus (IDDM; type I) is a chronic disease stemming from little or no insulin production and elevated blood glucose levels. Hyperglycemia causes cellular damage by many mechanisms including osmotic stress. IDDM is associated with an extensive list of late complications involving nearly every tissue. One major concern to young and aging diabetics is the association of IDDM with decreased bone mass and osteoporosis. These conditions are associated with an increase in hip and vertebrae fracture which can be debilitating and increase mortality.

Based on the literature, a major factor contributing to bone loss in diabetes is a decrease in osteoblast function and ability to differentiate, we therefore hypothesize that osteoblasts respond to increasing extracellular glucose concentration (as seen in diabetes) through an osmotic response pathway which influences gene expression and cell

phenotype. We anticipate that protein kinases such as protein kinase C (PKC) and mitogen activated protein kinases (MAPK) could be activated and result in changes in AP-1 and CRE transactivation. To experimentally test this hypothesis, four aspects of osteoblast responsiveness were examined: 1) phenotype and gene expression, 2) signal transduction, 3) transcription factor modulation and 4) *in vivo* vs *in vitro* effects.

Our findings, both *in vitro* and *in vivo,* are the first to demonstrate an increase in expression of *c-jun* and collagen I as well as the alkaline phosphatase activity in response to treatment with a physiologically relevant concentration of sugar (22 mM). In contrast, extracellular mineralization is decreased under these conditions and is potentially the result of osteoblast differentiation being halted at mid stage. The use of mannitol treatment and glucose uptake studies supports the novel concept that this response is osmotic in nature. The present studies also demonstrate that osteoblast responsiveness to moderate osmotic stress leads to an increase in AP-1 and CRE transactivation and DNA binding marked by the increased presence of c-Jun and ATF-2 in the binding complex. Inhibitor studies demonstrate that both PKC and p38 MAP kinase are involved in this response.

Findings from these studies contribute to an understudied area of research, the molecular and cellular pathways involved in diabetes induced bone loss. With an increasing diabetic and elderly population this information is important for developing counter measures to diabetes and possibly age related bone loss, perhaps by using drugs directed at signaling pathways and transcription factors affected by elevated glucose.

This work is dedicated to my lovely wife Lamia, my son Alexander, and Abraham, the new blessing we are expecting in December 2001.

I would also like to thank my parents, my two sisters Shaza and Nada, and my brother Ahed for their constant encouragement and belief in me.

ACKNOWLEDGMENTS

I would like to thank my committee members Drs. Laura R. McCabe, Patrick F. Dillon, Donald B. Jump, Seth R. Hootman, Laurie K. McCauley, and William S. Spielman. I am forever grateful to my mentor Dr. Laura McCabe for all the encouragement, guidance and support she gave me throughout my graduate research work. Furthermore, I would like to thank Drs Patrick Dillon, Donald Jump, and Seth Hootman for giving me some of their valuable time, comments and great discussions.

I would like to thank all my laboratory friends for their support and help, especially Shira Bassly, Brian Coates, Regina Irwin, Chris Ontiveros, Ruijie Shu and Christine Stell. My special thanks as well for my friends in the physiology department, Julia Busik, Nara Parameswaran, Maria Pino, Robert Reid, Panayiotis Vacratsis,

Last but not least, my thanks go to the department personnel, especially Sharon Shaft for her continuous great help.

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KEY TO ABBREVIATIONS

AP-1	Activating protein 1
AQP	Aquaporin
AR	Aldose reductase
ATF	Activating transcription factor
BGT	Betain/γ-amino-n-butyric acid transporter
COL	Collagen
CRE	cAMP response element
CREB	cAMP response element binding proteins
CREM	cAMP response element modulator
ECF	Extracellular fluid
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinases
FN	Fibronectin
Glut	Glucose transporter
ICF	Intracellular fluid
ICAM	Intracellular adhesion molecule-1
IDDM	Insulin dependent diabetes mellitus
JNK	c-Jun NH_2-terminal kinases
LUC	Luciferase
MAPK	Mitogen activated protein kinase
NAD	Nicotinamide adenine dinucleotide
PKC	Protein kinase C
PTH	Parathyroid hormone
RVI	Regulatory volume increase
SMIT	Na^+-dependent myo-inositol transporter
TauT	Taurine Transporter

I. Introduction

Poorly controlled or untreated type I diabetes mellitus, which affects over 5 million people in the United States, is characterized by hyperglycemia and is associated with decreased bone mass (Hui et al., 1985), impaired skeletal development (Levin et al., 1976), increased fracture rate (Bouillon, 1991; Meyer et al., 1993), and osteoporosis (Auwerx et al., 1988; Krakauer et al., 1995). With the longer life span of diabetics, understanding the mechanisms responsible for bone loss is critical for developing countermeasures to its deleterious effects.

Thomas et al. (1996) have identified GLUT1 and GLUT3 as the primary glucose transporters in osteoblast like cells (Thomas et al., 1996 a; Thomas et al., 1996 b). Therefore, osteoblasts have a limited capability to absorb glucose due to the lack of the high K_m glucose transporters. This means that at euglycemic state, when glucose is 3-5.5 mM, these transporters are fully saturated and have already reached their maximal capacity to transport glucose. In poorly controlled or untreated type I diabetes mellitus, blood glucose levels are constantly high. Consequently, this could place osteoblasts under osmotic stress if glucose transporters at the cell membrane number remain constant in number.

Cells can respond to different extracellular inputs from their environment by activating protein kinases. These complex networks allow amplification of extracellular signals. Activation of more than one selected pathway can mediate a diversity of cellular responses. For example, osmotic stress can activate the PKC signaling pathway (Schaffler et al., 2000) as well as mitogen-activated protein (MAP) kinase cascades.

Under physiologically relevant increases in extracellular sugar p38 kinase and PKC in particular have been shown to be activated in different cell culture systems (Kreisbeg and Kreisberg, 1995; Watts et al., 1998; Roger et al., 1999).

Both PKC and p38 pathways can potentially influence AP-1 levels and transactivation. Angel and Karin (1991) have shown that *c-jun* transactivation is increased in response to an increase in PKC activity. Modulation of Fos and Jun family member expression in bone by steroid hormones, overexpression, or targeted gene ablation has implicated c-Fos and other Fos and Jun related proteins in the regulation of bone tissue formation (Lian et al., 1991).

Activating transcription factor 2 (ATF-2) is a substrate of p38 and other stress-activated MAP kinases. It is a DNA-binding protein that forms a homodimer or heterodimer with c-Jun, binds to CRE and stimulates CRE dependent transcription of genes (Kawasaki et al., 2000). Van Dam et al. (1995) have shown that *c-jun* induction can occur by ATF-2 activation through the phosphorylation of p38. Increased activation and expression of transcription factors ultimately leads to gene modulation.

Based on our preliminary data and literature, we hypothesize that osteoblasts respond to increasing extracellular glucose concentration (as seen in diabetes) through an osmotic response pathway which influences gene expression and phenotype. Activation of protein kinase C (PKC), modulation of the mitogen activated protein kinase (MAPK) and changes in AP-1 and CRE transcription factors are involved in these processes.

The aim of this thesis is to study the effect of moderate elevation of extracellular glucose on osteoblasts, the bone forming cells. It is divided into six chapters. Chapter I includes a short introduction and states the major aims and hypothesis. A review of the

relevant past and current literature in Chapter II summarizes our current understanding of osmotic stress and the cellular response to hyperosmolarity. As this work is focused on physiologically relevant hyperosmolarity as seen in diabetes, I reviewed the effect of diabetes on bone and the role it plays as a hyperosmotic producing condition which leads to the activation PKC and MAP kinase. The last part of this review summarizes the current knowledge about osteoblasts and the importance of different transcription factors in bone development.

Chapter III focuses on the effect of increased extracellular glucose on osteoblast phenotype. This research project is designed to test the hypotheses that: (1) elevated extracellular glucose influences gene expression in osteoblasts, (2) glucose mediates AP-1 expression, and (3) PKC activation is required for the modulation of AP-1 and collagen I. Chapter IV further addresses the role of AP-1 in osteoblast responsiveness to elevated extracellular glucose. We tested the hypotheses that: (1) osteoblasts elicit an osmotic response to elevated extracellular glucose, (2) *in vivo* studies are consistent with *in vitro* data, and (3) the use of AP-1 dominant negative abolishes both the AP-1 modulation and gene expression changes in response to elevated extracellular glucose.

Chapter V focuses on the mechanism of the osteoblast osmotic response to elevated extracellular glucose. Based on the literature and our previous results this section addresses the hypotheses that: (1) MAP kinase activation is part of the osteoblast response to osmotic stress, and (2) downstream transcription factors of MAP kinase, such as ATF-2, are activated. The final summary and the conclusions of this work are summarized in Chapter VI.

Diabetes affects over 5 million people in the United States. Bone loss resulting from diabetes is of major concern given the long-term survival of diabetic patients. Yet, little is known in this area. Results from this project contribute to the understanding of the cellular and molecular mechanisms regulating osteoblast phenotype in response to diabetes. This information will aid in the development of drugs, directed at pathways affected by elevated extracellular glucose levels, which can be used to increase bone formation in diabetics and perhaps in the elderly. Such therapies can be used to reduce and possibly prevent the detrimental effects of osteoporosis.

II. Literature Review

1. Hyperosmolarity

Water is the most abundant constituent in the body, comprising approximately 60 percent of body weight in men and 50 percent in women. Total body water is distributed into two major compartments, 55 to 75 percent is intracellular [intracellular fluid (ICF)], and 25 to 45 percent is extracellular [extracellular fluid (ECF)]. The ECF is further subdivided into intravascular (plasma water) and extravascular (interstitial) space. Water in the body is the fluid in which important salts and proteins are dissolved. The concentration of these solutes or particles within a fluid is known as osmolality and is expressed as milliosmoles per kilogram of water (mOsmol/kg H_2O). Water crosses cell membranes to achieve osmotic equilibrium between extracellular and intracellular compartments. Disparities in cellular membrane permeability and the presence of transporters and active pumps allow extracellular and intracellular solutes to markedly differ while still maintaining equal particle concentration and osmolality. Intracellular particle concentration is normally constant while extracellular particle concentration can significantly change. This causes water to move into or out of cells due to alterations in extracellular osmolality resulting in a cellular volume change.

1.1 Osmotic Adaptation

Many cells studied to date respond to induced volume changes by modulating membrane transport and/or metabolic pathways that alter the concentration of intracellular solutes. The first adaptive process occurring in response to extracellular

hypertonicity-induced cell shrinkage is a regulatory cell volume increase (RVI). The RVI results from the stimulation of ion transporters such as the Na^+-K^+-$2Cl^-$ cotransporter pathway (Lytle and Forbush, 1996) and Na^+-H^+ exchanger (Grinstein et al., 1986). These transporters have been reviewed extensively in Parker, (1993). This immediate response increases the intracellular ion content within minutes and partially restores the cellular volume from the initial cell shrinkage (Parker, 1993; Sun et al., 1990). A second adaptive mechanism occurs within hours to days and results in the induction of genes encoding proteins involved in the accumulation of intracellular "compatible osmolytes". The general classes of organic osmolytes are sugars, polyols, amino acids, methylamines, and urea. The accumulation of osmolytes is facilitated by the induction of specific osmolyte transporters such as: betain/γ-amino-n-butyric acid transporter (BGT_1) for betaine (Yamauchi et al., 1992); the Na^+-dependent myo-inositol transporter (SMIT) for inositol (Kwon et al., 1992); and the taurine transporter for the amino acid taurine (Uchida et al., 1992). In some cases, enzymes that can produce osmolytes can be increased such as aldose reductase (AR) which catalyzes the NADPH-mediated reduction of aldehydes such as D-glucose to sorbitol (Bohren et al., 1989; Garcia-Perez et al., 1989). These osmolytes increase intracellular osmolality and restore isotonicity (equal intracellular and extracellular osmolality).

1.2 The effect of hyperosmolality

The normal plasma osmolality is about 300 mOsmol/kg H_2O and is kept within a narrow range by mechanisms capable of sensing a 1 to 2 percent change in tonicity. However, a hyperosmolar blood milieu can commonly occur in a number of clinical

conditions such as diabetes mellitus, uremia, and hypernatremia. The body and it's organs must adapt to this new environment. This adaptation response can lead to pathological damage and altered organ function potentially as a result of signaling pathway activation leading to modulation of gene expression.

1.2.1 Osmotic stress and signal transduction

Protein kinase C (PKC) and mitogen-activated protein (MAP) kinase cascades are important intracellular signal-transduction pathways that have been demonstrated to be activated in response to different extracellular stimuli including changes in osmolality. PKC is regulated by two sequential mechanisms: 1) phosphorylation triggered by 3-phosphoinositide-dependent kinase (PDK)-1 and 2) binding to the lipid second messenger diacylglycerol. Each mechanism regulates the structure, subcellular localization, and function of PKC (Dempsey et al., 2000). MAP kinases are serine/threonine kinases activated via a cascade of kinases involving a sequential phosphorylation of two kinases (MAP kinase kinase kinase and MAP kinase kinase), which activate a MAP kinase via a dual phosphorylation on threonine and tyrosine residues (Kolch, 2000; Widmann et al., 1999) (Figure.1). In mammalian cells, the MAP kinase family contains three major subgroups responding to distinct extracellular stimuli: extracellular signal-regulated kinases 1 and 2 (ERK); c-Jun NH_2-terminal kinases (JNK, also known as stress-activated protein kinases); and p38 kinases (also known as stress-activated protein kinases). The ERK pathway is principally activated by growth factors, integrin-matrix interaction, and hormones, while JNK and p38 are strongly activated by inflammatory cytokines, ultraviolet light, and hypertonic stress (Chen et al., 1996; Igarashi et al., 1999).

Activation of specific signaling pathways in response to hyperosmolality can mediate the required specific cellular adaptive response. Both PKC and p38 pathways have been demonstrated to be co-activated in response to osmotic stress in several cell systems. The target genes and importance of these pathways in response to osmotic stress is not completely understood. However, a study by Schaffler et al., (2000) demonstrates that inhibition of both PKC and p38 pathways can ameliorate hyperglycemic and hyperosmotic induced vascular dysfunction *in vivo* suggesting that both pathways are involved in this pathology. Past studies examining the mechanisms of cellular responsiveness to osmotic stress have focused principally on the kidney and the responses to hyperosmolar conditions above 600 mOsm. More recently a variety of cell systems have been shown to respond to physiologically relevant hyperosmolality as seen in clinical conditions such as diabetes mellitus.

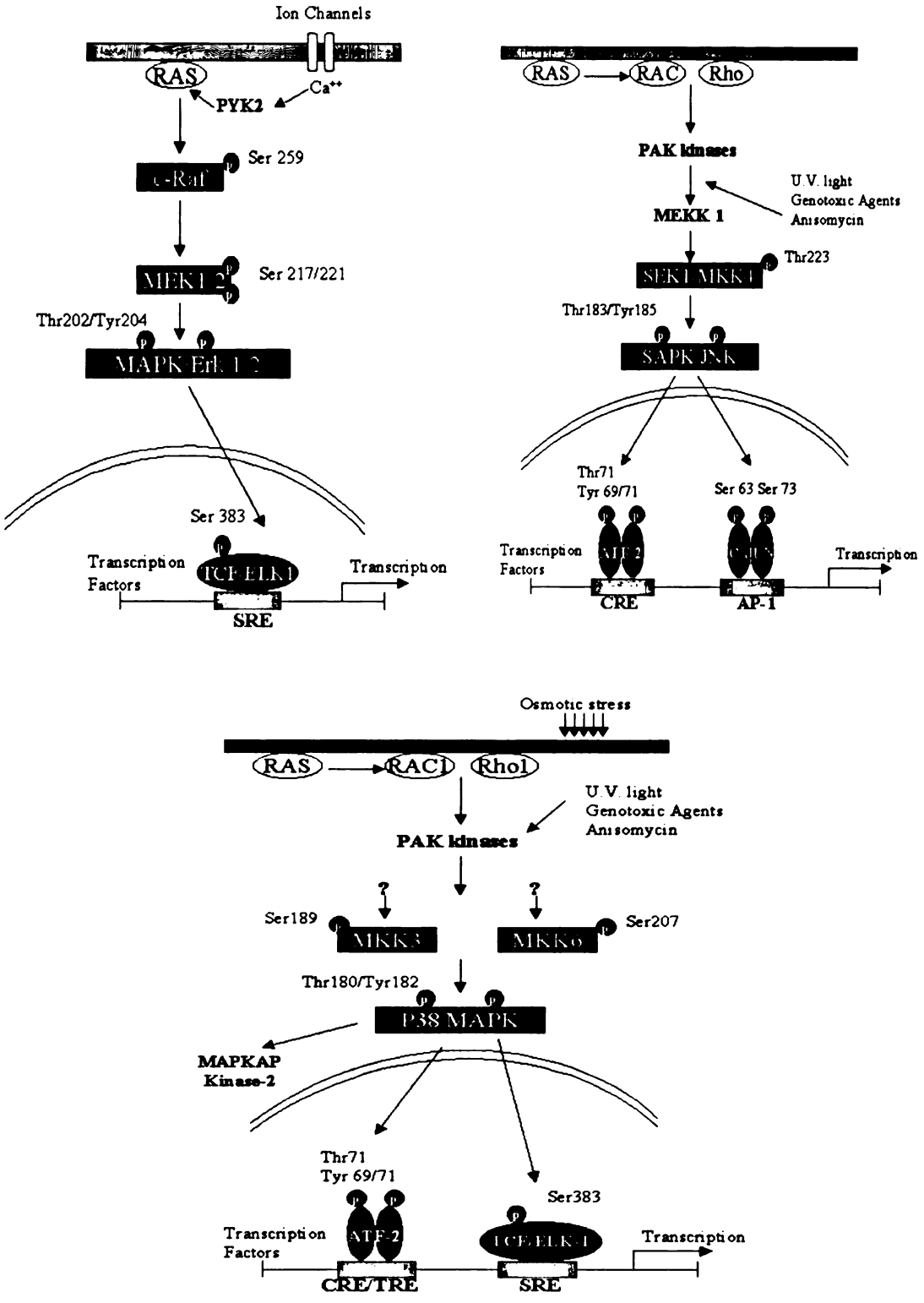


Fig. 1. **Illustration of the ERK, JNK and p38 MAPK kinase pathways.**

1.2.2 The Kidney and Hypertonicity

During diuresis and antidiuresis, the kidney medulla is exposed to large fluctuations of interstitial osmolarity (Knepper, 1996) which challenge cell volume constancy. Cells of the medullary thick ascending limb of Henle (MTAL) are of special interest, since they are the major contributor to the generation of the renal cortico-papillary osmotic gradient allowing urinary concentration in animals. Hyperosmolarity studies in kidney have been conducted on renal medullary cells subjected to a very high osmolar environment, 600 mOsm, consistent with the physiologic osmolarity that these cells are exposed to *in vivo*. Studies of cellular responses to high levels of hypertonicity implicate the activation of all or some of the mitogen-activated protein (MAP) kinase family members (Rosette and Karin, 1996; Galcheva-Gargova et al., 1994; Itoh et al., 1994; Terada et al., 1994; Zhang and Cohen, 1996; Berl et al., 1997; Watts et al., 1998). In MTAL cells Watts et al. (1998) demonstrated that hyperosmolality (600 mOsm) results in the rapid (within 5 minutes) and sustained (up to 60 minutes) activation of p38 kinase. ERK was also activated in these cells peaking at 15 minutes. The lack of responsiveness to treatment of MTAL cells with hyperosmolar levels of urea, a compound that rapidly enters the cell before significant volume changes can occur, demonstrates that cell shrinkage rather than extracellular hyperosmolality is critical for the activation of both ERK and p38 kinases in response to high osmolar environment (Roger et al., 1991). Activation of p38, in particular, is critical for regulatory volume increases in medullary cells. Studies done by Sheikh-Hamad et al., (1998) using renal medullary epithelial cells (MDCK) demonstrate that a specific p38 kinase inhibitor,

SB203580, can block the induction of betaine transporter mRNA in response to hyperosmotic media (600 mOsm) and prevent regulatory volume increases.

Recent clinical trials have shown that the degree of hyperglycemia is an important predictor of diabetic renal complications. Results of *in vivo* studies have given supportive evidence that increased ambient glucose concentrations exert an important influence on extracellular matrix in renal diabetic pathology (Ziyadeh et al., 1993). As diabetic nephropathy is characterized by the early appearance of glomerular hypertrophy, many researchers have started to look into the effect of moderate osmolality increase on other renal cells such as mesangial cells that are not usually exposed to high osmolar environment of 600 mOsm. Elevating extracellular glucose or mannitol (a non-metabolizable sugar) by 16-25 mM mimics the osmotic effect of diabetes mellitus, marked by a 16-25 mOsm increase in blood osmolarity, results in increased mesangial gene expression. In particular, extracellular matrix and attachment proteins expression has been reported to be increased (Park et al., 2000; Nahman et al., 1992; Kreisbeg and Kreisberg, 1995). The progressive accumulation of extracellular matrix (fibronectin, laminin, and collagen IV) in the glomerular mesangium leads to glomerulosclerosis and fibrosis (Mauer et al., 1994; Sharma et al., 1997; Ziyadeh et al., 1993). Both p38 and PKC are implicated in these responses (Morrisey et al., 1999; Ingram et al., 1999; Kreisbeg and Kreisberg, 1995).

1.2.3 The Brain and Hypertonicity

The kidney is not the only organ in the body which is sensitive to extracellular osmotic fluctuation. The brain with its different cellular components is also affected.

Osmoreceptors sensitive to changes in the osmolality of circulating blood have been located in the basal forebrain and their activation is responsible for the secretion of vasopressin (Stricker, 1999). When exposed to a hyperosmotic medium, brain cells, similar to many other cells in the body accumulate osmolytes and thus achieve osmotic equilibrium with the medium while maintaining their volume. For example, when astrocyte primary cultures are exposed to hyperosmolar medium they shrink rapidly and then slowly regain their initial volume after several hours (Bitoun and Tappaz, 2000). The mRNA levels of osmolyte transporters (taurine - TauT, myo-inositol - SMIT and betaine - BGT1) showed significant and comparable increases after a four hour exposure and return to near normal levels after 24 hours. When taurine was added to the hyperosmotic medium, cell volume recovery was greatly accelerated and the osmo-induced overexpression of TauT, SMIT and BGT1 mRNA was prevented (Bitoun and Tappaz, 2000). Taurine is an amino acid mainly known for its involvement in cell volume regulation; it is one of the major inorganic osmolytes used by cells to compensate for changes in extracellular osmolarity. In the supraoptic nucleus, taurine is highly concentrated in astrocytes, and released in an osmodependent manner through volume-sensitive anion channels (Saransaari et al., 2000). Via its agonist action on neuronal glycine receptors, taurine is likely to contribute to the inhibition of neuronal activity induced by hypotonic stimuli. This inhibitory influence would complement the intrinsic osmosensitivity of supraoptic neurons, mediated by excitatory mechanoreceptors activated under hypertonic conditions (Hussy et al., 2000). These observations demonstrate the role taurine plays in the regulation of neural cell volume to that of whole body fluid balance.

1.2.4 Blood vessels and Hypertonicity

The water in the blood and plasma is what constitute the intravascular extracellular fluid. Therefore, a rise in the osmolality of the blood is considered an extracellular hyperosmolality. This increase affects both cells circulating in the blood as well as the cells lining the blood vessels. Similar to renal and brain cells, cell shrinkage due to osmotic stress modulates leukocyte attachment and endothelial extracellular matrix proteins through the activation of protein kinase pathways. Hyperosmolality for example, affects neutrophils partially by altering surface expression of adhesion molecules, CD11b and L-selectin (Rizoli et al., 1998). Volume reduction in neutrophils results in an increase in tyrosine phosphorylation and activation of p38. Similar to renal medullary cells, the trigger for this response is cell shrinkage and not an increase in osmolarity, ionic strength, or intracellular pH (Rizoli et al., 1999). The fact that cell shrinkage is the triggering event for the osmotic response led Rizoli et al. (2000) to investigate if osmotically induced cytoskeletal changes might be related to this response. They found that osmotic stress provoked a twofold increase in F-actin, induced the formation of submembranous F-actin ring, and abolished depolymerization that normally follows agonist-induced actin assembly. These results suggest that cytoskeletal remodeling is a key component in the neutrophil response to hypertonicity. Monocytes and macrophages in the blood are also targets for hyperosmolality. They use betaine and myoinositol "compatible organic osmolytes" when exposed to osmotic stress. Interestingly, p38 MAP kinase is found to be involved in the hyperosmolarity-induced upregulation of the osmolyte transporters BGT-1 and SMIT (Denkert et al., 1998).

Vascular endothelial cells are not an exception when it comes to responding to osmotic stress. Hypertonic treatment with physiologically relevant levels of glucose or mannitol significantly increased the level of p38 activity. Other osmotic inducing agents were used and the study concluded that different agents induce MAP kinase activation differently. Urea did not affect the level of induction of the MAP kinase isoforms which implies that cell shrinkage maybe an important component of hyperosmolality-induced MAP kinase phosphorylation (Duzgun et al., 2000).

From all the above we can conclude that many cells in the body respond to moderate increases in extracellular osmolarity as seen in diabetes where blood glucose can be as high as 22mM. This response seems to be largely driven by a change in cell volume "shrinkage" and leads to activation of various signal transduction pathways resulting in changes in gene expression.

2. Bone as a tissue

Bone is a specialized connective tissue that makes up, together with cartilage, the skeletal system. These tissues serve three functions: mechanical, protective, and metabolic. In bone, as in all connective tissues, the fundamental constituents are the cells and the extracellular matrix. The latter is particularly abundant in these tissues and is composed of collagen fibers and noncollagenous proteins.

2.1 Bone Matrix and Mineral

Bone is formed by collagen fibers (type I, 90% of the total protein), usually oriented in a preferential direction, noncollagenous proteins and a calcium phosphate

matrix termed hydroxyapatite. Spindle-or plate- shaped crystals of hydroxyapatite are found on the collagen fibers, within them, and in the ground substance. They tend to be oriented in the same direction as the collagen fibers. The ground substance is primarily composed of glycoproteins and proteoglycans. These highly anionic complexes have a high ion-binding capacity and are thought to play an important part in the calcification process and the fixation of hydroxyapatite crystals to the collagen fibers.

The preferential orientation of the collagen fibers alternates in adult bone from layer to layer, giving to this bone a typical lamellar structure, best seen under polarized light or by electron microscopy. This fiber organization allows the highest density of collagen per unit volume of tissue. The lamellae can be parallel to each other if deposited along a flat surface (trabecular bone and periosteum), or concentric if deposited on a surface surrounding a channel centered on a blood vessel (haversian system). However, when bone is being formed very rapidly (during development and fracture healing, or in tumors and some metabolic bone diseases), there is no preferential organization of the collagen fibers; this type of bone is called woven bone as opposed to lamellar bone.

2.2 Cellular organization within the bone matrix –the osteocyte

The calcified bone matrix is not metabolically inert, and cells (osteocytes) are found embedded deep within the bone in small osteocytic lacunae (25,000/mm^3 of bone) (Aarden et al., 1994). Osteocytes were once bone-forming cells, osteoblasts, that became trapped in the bone matrix that they produced, which later became calcified. They nevertheless express some specific membrane proteins. These cells have numerous and long cell processes rich in microfilaments, which are in contact with cell processes from

other osteocytes or with processes from the cells lining the bone surface. These processes are organized during the formation of the matrix and before its calcification; they form a network of thin canaliculi permeating the entire bone matrix (Aarden et al., 1994). Between the osteocyte's plasma membrane and the bone matrix itself is the periosteocytic space. This space exists both in the lacunae and in the canaliculi, and it is filled with extracellular fluid.

2.3 The osteoblast and bone formation

The osteoblast is the bone-lining cell responsible for the production of the matrix constituents (collagen and ground substance). It originates from a local mesenchymal stem cell (bone marrow stromal stem cell or connective tissue mesenchymal stem cell). These precursors, with the right stimulation, undergo proliferation and differentiate into preosteoblasts and then into mature osteoblasts. Osteoblasts never appear or function individually but are always found in clusters of cuboidal cells along bone surface (~100 to 400 cells per bone-forming site). At the light microscope level, the osteoblast is characterized by a round nucleus at the base of the cell (opposite the bone surface), a strongly basophilic cytoplasm, and prominent Golgi complex located between the nucleus and the apex of the cell. Osteoblasts are always found lining the layer of bone matrix that they are producing, before it is calcified (called, at this point, osteoid tissue). Osteoid tissue exists because of a time lag between matrix formation and its subsequent calcification (the osteoid maturation period), which is approximately 10 days (Pockwinse et al., 1995). Behind the osteoblast can usually be found one or two layers of cells: activated mesenchymal cells and preosteoblasts. At the ultrastructural level, the

osteoblast is characterized by (a) the presence of an extremely well-developed rough endoplasmic reticulum with dilated cisternae and a dense granular content, and (b) the presence of large circular Golgi complex comprising multiple Golgi tasks (Ozawa et al., 1994). Cytoplasmic processes on the secreting side of the cell extend deep into the osteoid matrix and are in contact with the osteocyte processes in their canaliculi. Junctional complexes (gap junctions) are often found between the osteoblasts (Ozawa et al., 1994). The plasma membrane of the osteoblast is characteristically rich in alkaline phosphatase (the concentration of which in the serum is used as an index of bone formation) and has been shown to have receptors for parathyroid hormone but not for calcitonin (Nijweide et al.,1986). Osteoblasts also express steroid receptors for estrogens and vitamin D3 in their nuclei, as well as several adhesion molecules (integrins) and receptors for cytokines. Toward the end of the secreting period, the osteoblast becomes either a flat lining cell or an osteocyte.

In culture, like *in vivo*, osteoblasts form a bone tissue-like organization by undergoing three stages of development: proliferation, extracellular matrix maturation, and extracellular matrix mineralization (Quarles et al., 1992; Owen et al., 1990). After 4-5 weeks in culture, mineralized bone nodules are visible in the culture dish. During each stage of development, specific subsets of genes are sequentially expressed or repressed (see Figure 2), i.e.: proliferation – histone H4 and collagen I, extracellular matrix maturation – alkaline phosphatase, and mineralization – osteocalcin and osteopontin. The regulation of gene expression in developing osteoblasts occurs predominantly at the transcriptional level. This suggests that transcription factors play a major role in regulating osteoblast phenotype and therefore bone formation.

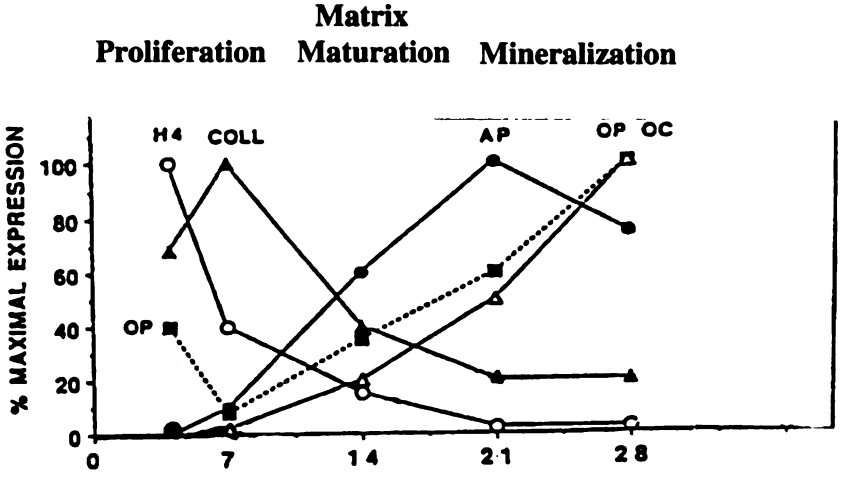


Figure 2. **Osteoblast developmental sequence.** Temporal expression of osteoblast cell growth and phenotype related genes during 28 days of culture. Cells were harvested on the indicated days and total RNA was analyzed by northern blot analysis. In the proliferative period, histone (H4), and collagen I (COLL) are maximally expressed. The peak level of alkaline phosphatase (AP) mRNA represents the ECM maturation period. In the mineralized period, osteopontin (OP) and osteocalcin (OC) reach their peak mRNA levels. A similar graph would also represent transcription rates, obtained from nuclear run on assays, suggesting that transcription factors are important for this orchestration of gene expression.

2.4 The osteoclast and bone resorption

The osteoclast is a giant multinucleated cell, containing four to 20 nuclei. It is usually found in contact with a calcified bone surface and within a lacuna that is the result of its own resorptive activity. It is possible to find up to four or five osteoclasts in the same resorptive site, but there usually are only one or two (Roodman, 1996). Under the light microscope, the nuclei appear to vary within the same cell: some are round and euchromatic, and some are irregular in contour and heterochromatic, possibly reflecting the asynchronous fusion of mononuclear precursors. The cytoplasm is "foamy" with vacuoles. The zone of contact with the bone is characterized by the presence of a ruffled border with dense patches on each side (the sealing zone)(Baron, 1993).

Characteristic features of osteoclast are the abundant Golgi complexes disposed around each nucleus, the mitochondria, and the transport vesicles loaded with lysosomal enzymes. The most prominent features of the osteoclast are the deep foldings of the plasma membrane in the area facing the bone matrix. The ruffled border in the center is surrounded by a ring of contractile proteins (sealing zone) that serve to attach the cell to the bone surface, thus sealing off the subosteoclastic bone-resorbing compartment (Suda et al., 1992). The attachment of the cell to the matrix is performed by integrin receptors, which bind to specific sequences in matrix proteins. The plasma membrane in the ruffled border area contains proteins that also found at the limiting membrane of lysosomes and related organelles, and a specific type of electrogenic proton adenosine triphosphatase (ATPase) involved in acidification. The basolateral plasma membrane of the osteoclast is highly and specifically enriched in (Na^+, K^+) ATPase, HCO_3^-/Cl^- exchangers and Na^+/H^+ exchangers (Baron, 1999).

2.5 Bone remodeling

Bone formation by osteoblasts and resorption by osteoclast occurs along the surface of bone, mainly at the endosteal surface, and results in bone remodeling. This activity is involved in bone growth and turnover and does not occur at random. Bone formation and resorption are either part of the process of bone development and growth or part of the turnover mechanism by which old bone is replaced by new bone. In the normal adult skeleton, (i.e. after the period of development and growth), bone formation occurs only where bone resorption has previously occurred (Marks and Hermey, 1996). The sequence of events at the remodeling site involves osteoclast activation, bone resorption and subsequently bone formation. During the intermediate phase between resorption and formation (the reversal phase) a cement line is formed that marks the limit of resorption and acts to cement together the old and the new bone. The duration of these various phases has been measured. The complete remodeling cycle at each microscopic site takes about 3 to 6 months.

3. Influence of diabetes on bone

Insulin dependent diabetes mellitus (IDDM; type I) is a chronic disease stemming from little or no insulin production and elevated blood glucose levels. IDDM is associated with an extensive list of late complications involving nearly every tissue. One major concern to young and aging diabetics is the association of IDDM with osteoporosis (Auwerx et al., 1988; Krakauer et al., 1995), decreased bone mass (Hui et al., 1985), impaired skeletal development (Levin et al., 1976) and increased fracture rates (Bouillon, 1991; Meyer et al., 1993). Diabetic bone disease was first recognized at the beginning of

the twentieth century (Morrison and Bogan, 1927) and described as retardation of bone development and bone atrophy in children with long-standing diabetes. Berney, (1952) also described the coexistence of diabetes and osteoporosis. Since then, diabetic bone and mineral homeostasis have been studied in both humans and experimental animals. Studies measuring bone loss by radiogrammetry and photon absorptometry demonstrate that both male and female diabetics are vulnerable to this outcome (Levin et al., 1976).

A recent clinical study was done to evaluate the prevalence and severity of osteopenia in patients with IDDM. It showed that 67% of the diabetic men and 57% of the diabetic women suffered from osteopenia (a bone mineral density BMD $\geq 1 < 2.5$ standard deviation of the population mean) of the femoral neck and/or lumbar spine (Kemink et al., 2000). Fourteen percent of the male patients, but none of the female patients, met the criteria for osteoporosis (BMD $\geq$ 2.5 SD). Analysis of bone formation and resorption markers in the blood demonstrate that at least in male patients with IDDM, osteopenia is the consequence of a lowered bone formation with a predominance of bone resorption over formation (Kemink et al., 2000). These effects are even more pronounced in diabetic rats. Verhaeghe et al. (1990) showed that long standing diabetes in BB rats results in severe low-turnover osteoporosis probably related to decreased osteoblasts recruitment and/or function. When titanium alloy implants are placed on normal and diabetic rat tibia for fourteen days, diabetic rats demonstrated significantly less osseointegration than control (McCracken et al., 2000).

Several hypotheses have been proposed as possible pathogenic mechanisms of diabetic osteopenia including primary disturbances in calcium absorption and secretion or vitamin D metabolism (Raskin et al., 1978; Schneider and Schedl, 1972; Spencer et al.,

1980). However, histochemical studies suggest that osteoblasts are markedly affected by diabetes. Specifically, osteoblast number/surface is reduced (cuboidal osteoblasts are replaced by bone lining cells) and osteoid surfaces are markedly decreased with no reduction in osteoclast number (Verhaeghe et al., 1990; Sasaki et al., 1991). These parameters do not correlate with bone loss resulting from low vitamin D or calcium levels, and they do not occur in semistarved animals (Shires et al., 1980). Thus, diabetes associated malnutrition and altered calcium and vitamin D metabolism cannot account for the marked changes in bone.

Data from clinical, morphologic, and biochemical investigations has established that hyperglycemia is a major metabolic consequence of diabetes and a major factor in the development of chronic diabetic complications (Brownlee et al., 1984). In bone however, most studies have focused on the effects of insulin. For example, insulin treatment can stimulate bone mineralization in IDDM rats (Verhaeghe et al., 1992; Hough et al., 1981).

Studies in type II non-insulin dependent diabetes mellitus also suggest a positive effect of elevated insulin levels which are strong enough to overcome any negative influences of other factors (Haffner and Bauer, 1993; Krakauer et al., 1995). However, some animals still exhibit bone loss and increased fracture rate with NIDDM (Takeshita et al., 1993) and increased bone loss with insulin treatment of IDDM (Waud et al., 1994). Differences among these studies may result from difference in rat strain, differences in overall daily blood glucose and insulin levels, and/or changes in other serum factors (Taylor et al., 1987). These results suggest that modulation of the regulation of osteoblast

growth and differentiation is potentially a key component of the bone loss associated with diabetes.

To directly address the role of glucose on osteoblast function, an *in vitro* culture system can be used. Only a few studies have utilized this approach, but all have shown an influence of glucose on osteoblasts. For example, when human osteoblasts (MG-63 cells) are exposed to elevated glucose levels, both parathyroid hormone (PTH) and vitamin D responsiveness are impaired (Yoshida et al., 1995; Inaba et al., 1995). In addition, Terada et al. (1998) demonstrated that 7 day exposure of MG-63 cells to elevated extracellular glucose levels results in attenuated responsiveness to IGF-1 treatment. These findings demonstrate that glucose can influence osteoblast responsiveness to extracellular signals. However, the mechanism of these effects and consequences of high glucose treatment on the regulation of osteoblast differentiation, transcription factor levels, and gene expression remain unknown.

4. Diabetes and hyperosmolality

Diabetes is diagnosed by an elevated blood glucose level that is greater than 200 mg/dl (11 mM) at any random testing (Emancipator, 1999). Elevated extracellular glucose has been shown to cause significant cellular effects including: 1) nonenzymatic glycosylation of intracellular and extracellular proteins and DNA (Brownlee et al., 1984; Bucala et al., 1984; Locatto et al., 1993; McCarthy et al., 1997; Katayama et al., 1996), 2) modulation of cellular redox state (Hunt et al., 1990; Wolff and Dean, 1987), 3) changes in cell metabolic pathways such as activation of the polyol pathway (Inaba et al., 1995;

Gabbay, 1973; Larkins and Dunlop, 1992), and 4) activation of protein kinase C (Lee et al., 1994; Wolf et al., 1991; Craven et al., 1990; Ceolotto et al., 1999). Recently in rat osteoblast like cells (UMR 106-01) GLUT1 and GLUT3 glucose transporters have been identified (Thomas et al., 1996 a; Thomas et al., 1996 b). GLUT 1 and GLUT 3 are basal glucose transporters with a low K_m, 1-2 mM for GLUT1 and <1 mm for GLUT3 (Bell, 1991). The K_m in this case is the glucose concentration at which transport is half maximal. This means that at euglycemic state, when glucose is 3-5.5 mM, these transporters are fully saturated and have already reached their maximal capacity to transport glucose. In poorly controlled or untreated type I diabetes mellitus glucose level is constantly high. Consequently, this could place osteoblasts under osmotic stress and induce a combination of several mechanisms and signaling pathways, which contributes to the glucose induced complications of osteoblast function in diabetes.

5. Transcription Factors

Downstream targets of protein kinase pathways include activation of transcription factors and gene expression. For example, PKC has long been known to activate the binding and transactivation at AP-1 sites. This has been shown by the PKC activation following TPA treatment leading to an increase in the AP-1 consensus DNA binding on the TPA response element (TRE). AP-1 activation is involved in the regulation of the transcription of different genes such as collagen I and osteocalcin (Katai et al., 1992; McCabe 1996). MAP kinases have also been mostly attributed to the control of gene transcription via phosphorylation of nuclear transcription factors such as ELK, Jun and ATF-2. This eventually leads to gene modulation, which can play a part in the cellular

response to cell shape changes and osmotic stress. Hoffert et al. (2000) demonstrated that aquaporin-5 (AQP5), a water channel protein, is induced by hypertonic stress and this induction requires activation of extracellular signal-regulated kinase (ERK). In diabetic nephropathy, an excessive production of extracellular matrix (ECM) proteins by glomerular mesangial cells occurs. Ishida et al. (1999) demonstrates that MAP kinase may contribute to the overproduction of fibronectin (FN) in mesangial cells. This response was not only protein kinase mediated, but it also enhanced DNA-binding activity of AP-1 as well.

5.1 AP-1 family of transcription factors

The nuclear proto-oncogenes, c-Fos and c-Jun, are the proto-typical members of the AP-1 family of transcription factors whose members also include: Fos B, Fra-1, Fra-2, Jun B, and Jun D. All family members contain a leucine zipper and a basic region required for dimer formation and DNA binding, respectively (Kouzarides and Ziff, 1988). Fos and Jun proteins can form heterodimers, but only Jun proteins can form stable homodimers (Angel and Karin, 1991). Dimerization is a prerequisite for DNA binding to a consensus response element designated as an AP-1 site (5'-TGAg/cTCA-3'). Variations in core AP-1 sequences or flanking sequences can affect DNA binding affinities of specific dimer combinations (McCabe et al., 1996; Hadman et al., 1993; Ryseck and Bravo, 1991). Compositional changes and post-translational modifications in Fos and Jun dimer complexes can dramatically influence AP-1 DNA binding activity and transactivation potential (Gruda et al., 1994; Smeal et al., 1992). Therefore, modifications in Fos and Jun family member proteins and their levels define AP-1 dimer

subsets within the cell, and in turn could influence the transcription of genes with appropriate target AP-1 sites.

AP-1 members are critical in the response of osteoblasts to changes in extracellular stimuli. Modulation of Fos and Jun family member expression in bone by steroid hormones, overexpression, or targeted gene ablation has implicated c-Fos and other Fos and Jun related proteins in the regulation of bone tissue formation. For example, vitamin D (Candeliere et al., 1991), PTH (Clohisy et al., 1992; Lee et al., 1994; Koe et al., 1997), IGF (Merriman et al., 1990), BMP-2, (Ohta et al., 1992), or TGF-ß (Breen et al., 1994; Machwate et al., 1995) modulate c-fos and other fos/jun family member expression and at the same time influence the growth and differentiation of osteoblasts.

In osteoblasts, a significant change in the steady state levels (24 hours after feeding) of Fos/Jun family members and AP-1 DNA binding composition occurs during the onset of differentiation (McCabe et al., 1996; McCabe et al., 1995). Specifically, Fra-2 and Jun D are the predominant members present in differentiated osteoblasts at the mRNA and protein level as well as in AP-1 DNA binding complexes (McCabe et al., 1996). This is in marked contrast to proliferating cells that express Fos B, Fra-1, Fra-2, c-Jun, Jun B, and Jun D.

Although not well studied, AP-1-related sites are present in the promoters of many developmentally regulated genes abundant in osteoblasts. This group includes alkaline phosphatase, cyclin D1, collagen I, collagenase, osteopontin, and osteocalcin. Binding of different Fos and Jun dimers to unique AP-1 sites with different flanking sequences (yielding site selectivity of AP-1 pairs) and overlapping with other

transcription factor elements provides multiple options for selectively influencing the binding of factors necessary for basal and hormone stimulated gene expression. Knowing that Fos/Jun expression is modulated during growth and differentiation, it is possible to conceive of a system of gene regulation based on the presence of specific AP-1 dimers in a cell and their affinity for different AP-1 like sites within gene promoters.

5.2 CREB/ATF family of transcription factors

Cyclic AMP (cAMP)-responsive element (CREB) binding protein was originaly identified as a target of the cAMP signaling pathway, but studies on activation of immediate-early genes revealed that CREB is a target of other signaling pathways activated by diverse array of stimuli (Sheng et al., 1990). During the characterization of CREB, two other highly related products were identified: activating transcription factors (ATF) (Lee et al., 1987) and cAMP response element modulator (CREM) (Foulkes et al., 1991). Members of the CREB/ATF family bind to a consensus DNA sequence (TGACGTCA), have a similar DNA binding domain and form selective heterodimers with each other via the leucine zipper region. Although all CREB/ATF proteins share similarity in their bZip domains; subgroups of proteins share additional similarity in other regions. For example, ATF-1 (Hai et al., 1989), CREB (Gonzalez et al., 1989; Hoeffler et al., 1988) and CREM (Foulkes et al., 1991) are similar in regions that contain the phosphorylation sites. Similarly, ATF-2/CRE-BP1 (Maekawa et al., 1989) and ATF-3 (Gaire et al., 1990) share significant similarity in regions outside the bZip domain: the first 100 N-terminal residues and the last 13 C-terminal residues. It is possible that proteins within a given subgroup have closely related functions. Proteins between

subgroups, however, are completely different from each other outside the DNA binding domain, indicating that they may interact with different proteins or ligands and have different functions.

ATF-1 and CREB have been demonstrated to stimulate transcription in response to cAMP and calcium influx (Gonzalez and Montminy, 1989; Liu et al., 1993; Rehfuss et al., 1991), whereas ATF-2/CRE-BP1 has been demonstrated to stimulate transcription in response to viral induction resulting in phosphorylation of residues in their amino-terminal transactivation domain (Du et al., 1993). These sites are efficiently phosphorylated by stress activated protein kinases (SAPKs). Phosphorylation of ATF-2 by SAPK *in vitro* at one or more sites outside the transactivation domain increases the DNA binding activity of ATF-2 (Abdel-Hafiz et al., 1992). ATF-2 and c-Jun DNA binding activity is highly regulated in response to stress. This complex will then target various promoter sites, including the *jun*2TRE within the *c-jun* promoter (Morooka et al., 1995).

ATF-3 on the other hand, is not an activator; it represses transcription when bound to DNA (Chen et al., 1994). However, ATF-3 can heterodimerize with Jun proteins, and ATF-3/Jun heterodimers have been demonstrated to activate transcription (Hsu et al., 1992; Chu et al., 1994). Therefore, depending on the cellular context, ATF-3 may repress transcription as homodimers or activate transcription as heterodimers.

The role of CRE in the development of osteoblasts has not been fully characterized yet. But increasing evidence points to the importance of CRE in the development of bone and its response to different agents. Sakamoto et al. (1998) reported that in murine calvarial osteoblasts (MC3T3-E1) the amount of CREB protein is high

through all stages of osteoblast development and maximal in the proliferation stage. The degree of CREB phosphorylation reached high levels in the proliferation stage and early mineralization stage. In the early mineralization stage, most CREB bound to consensus CRE DNA binding sites was phosphorylated, while both phosphorylated and unphosphorylated CREB were bound in the proliferation stage. This leads to hypothesis that the phosphorylation of CREB may regulate the expression of genes defining the developmental sequence of MC3T3-E1 cells. ATF-2 mutant mice developed a defect in endochondral ossification at epiphyseal plates similar to human hypochondroplasia (Reimold et al., 1996).

Interestingly, the *c-fos* 5' regulatory region has several imperfect CRE sequences of which only one markedly causes cAMP-dependent *c-fos* promoter activation (Berkowitz et al., 1989). Several *in vivo* models have already identified Fos as an important player in bone biology, where overexpression causes neoplasms and collagenase producing bone tumors (Gack et al., 1994; Ruther et al., 1987) and Fos null mice exhibit osteopetrosis and disorganized bone growth (Grigoriadis et al., 1994). Pearman et al., (1996) showed that in normal osteoblasts, CREB participates in basal *c-fos* expression through CRE. Upon PTH exposure, PKA phosphorylates and activates CREB *trans*-activation functions. Increased Fos protein then participates in gene regulation in bone.

In summary, diabetes is associated with bone loss and decreased osteoblast function. Glucose levels are markedly increased in uncontrolled diabetes. The role of glucose in altering osteoblast phenotype has not fully been examined, especially with

regard to changes in osteoblast gene expression and differentiation. Important mechanisms by which glucose can influence osteoblast differentiation include altering cell metabolism, extracellular osmolality, signal transduction pathways, and transcription factor activities. Understanding the cellular and molecular mechanisms regulating osteoblast phenotype in response to diabetes might aid in the development of drugs, directed at pathways affected by elevated extracellular glucose levels, which can be used to increase bone formation in diabetics and perhaps in the elderly. Such therapies can be used to reduce and possibly prevent the detrimental effects of osteoporosis.

III. Extracellular Glucose Influences Osteoblast Differentiation and *c-jun* Expression

(This chapter was published in Journal of Cellular Biochemistry 79(2): 301-310 2000)

1. Abstract

Insulin dependent diabetes mellitus, marked by high blood glucose levels and no insulin secretion, is associated with decreased bone mass and increased fracture rates. Analysis of bone histology suggests that osteoblast phenotype and function are influenced by diabetes. To determine if elevated extracellular glucose levels could directly influence osteoblast phenotype we treated mouse osteoblasts, MC3T3-E1 cells, with 16.5 mM glucose and analyzed osteoblast gene expression. Collagen I mRNA levels significantly increased while osteocalcin mRNA levels decreased twenty-four hours after the addition of glucose. Expression of other genes, actin, osteopontin, and histone H4, was unaffected. Effects on collagen I expression were seen as early as one hour after treatment. c-Jun, an AP-1 transcription factor involved in the regulation of osteoblast gene expression and growth, was also modulated by glucose. Specifically, an increase in *c-jun* expression was found at 1 hour and maintained for 24 hours following glucose treatment. Treatment of osteoblasts with an equal concentration of mannitol completely mimicked glucose treatment effects on collagen I and *c-jun* expression, demonstrating that osmotic stress rather than glucose metabolism is responsible for the effects on osteoblast gene expression and phenotype. Additional studies using staurosporine and Ro-31-8220 demonstrate that protein kinase C is required for the glucose upregulation of collagen I and *c-jun*. Taken together our results demonstrate that osteoblasts respond to

increasing extracellular glucose concentration through an osmotic response pathway that is dependent upon protein kinase C activity and results in upregulation of *c-jun* and modulation of collagen I and osteocalcin expression.

2. Introduction

Insulin dependent diabetes mellitus (IDDM) is a chronic disease stemming from no insulin production and elevated blood glucose levels. IDDM manifest itself with many well-known complications such as vascular abnormalities, retinopathy, nephropathy, and neuropathy. Less well known, but of major concern, is the association of IDDM with osteoporosis (Auwerx et al., 1988; Krakauer et al., 1995), decreased bone mass (Hui et al., 1985), and increased fracture rates (Bouillon, 1991). Histochemical studies demonstrate a decreased number early stage and mature osteoblasts in diabetic bone (Verhaeghe et al., 1990; Sasaki et al., 1991). These changes cannot be accounted for by altered calcium and vitamin D metabolism (Shires et al., 1980). These findings suggest that modulation of the regulation of osteoblast growth and differentiation is potentially a key component of the bone loss associated with diabetes.

Diabetes is diagnosed by an elevated blood glucose level that is greater than 200 mg/dL (11 mM) at any random testing (Emancipator et al., 1999). Elevated extracellular glucose has been shown to cause significant cellular effects including: 1) nonenzymatic glycosylation of intracellular and extracellular proteins and DNA (Brownlee et al., 1984; Bucala et al., 1984; Locatto et al., 1993; McCarthy et al., 1997; Katayama et al., 1996), 2) modulation of cellular redox state (Hunt et al., 1990; Wolf and Dean 1987), 3) changes in cell metabolic pathways such as activation of the polyol pathway (Inaba et al., 1997;

Gabbay et al., 1973; Larkins and Dunlop 1992), and 4) activation of protein kinase C (Lee et al., 1989; Wolf et al., 1991; Craven et al., 1990; Ceolotto et al., 1999). These changes and perhaps other unidentified changes in signaling pathways and transcription factor activities can directly affect cell growth, differentiation, and function. Only a few studies have addressed the influence of glucose on osteoblast function. All suggest that an elevation in extracellular glucose levels can influence osteoblast function. For example, Katayama et al. (1996) have shown that culturing of osteoblasts on glycosylated collagen I substrates can variably suppress alkaline phosphatase activity and osteocalcin secretion. In addition to matrix effects, direct effects of glucose on osteoblast responsiveness and gene expression have been suggested. When human osteoblasts (MG-63 cells) are exposed to elevated glucose levels, parathyroid hormone (PTH), vitamin D and insulin-like growth factor (IGF-1) responsiveness are impaired (Yoshida et al., 1995; Inaba et al., 1995; Terada et al., 1998).

The AP-1 family of transcription factors has been shown to play an important role in the coupling of gene expression to changes in the environment (Morgan and Curran, 1991; Angel and Karin, 1991). This family includes c-Fos, Fos B, Fra-1, Fra-2, c-Jun Jun B, and Jun D. Fos:Jun heterodimers and Jun:Jun homodimers bind to a DNA consensus response element designated as an AP-1 site (5'-TGAg/cTCA-3') (Angel et al., 1991). Changes in the level of AP-1 member expression and post-translational modifications play an important role in regulating AP-1 DNA binding and transactivation (McCabe et al., 1996; Gruda et al., 1994; Smeal et al., 1992; Yamamoto et al., 1992).

Osteogenic hormones, bone strain and fracture, and Fos/Jun overexpression stimulate both AP-1 member expression and bone formation implicating this family of

transcription factors in the regulation of osteoblast growth and differentiation (McCabe et al., 1996; Hadman et al., 1993; Candeliere et al., 1991; Clohisy et al., 1992; Koe et al., 1997; Merriman et al., 1990; Breen et al.; 1994; Machwate et al., 1995; Ohta et al., 1991 & 1992; Mikuni-Takagaki Y,1999). This is not surprising seeing as osteoblasts must be highly responsive to extracellular changes such as bone strain and calcium and nutrient status within the body and must react immediately by modulating growth, differentiation, and gene expression. Exactly how osteoblasts respond to the stress of increased extracellular glucose is not known. Previous reports in other cell types suggest that protein kinase C activity, an activator of AP-1 expression, is elevated in response to glucose treatment (Lee et al., 1989; Wolf et al., 1991; Craven et al., 1990; Ceolotto et al., 1999). Furthermore, Kreisberg et al. (1994) have shown that 1 hour after glucose treatment mesangial cells have increased *c-fos* and *c-jun* expression, suggesting that AP-1 may play a role in cellular responsiveness to glucose. These findings led us to speculate that diabetes associated changes in osteoblast phenotype could at least in part be the result of altered AP-1 family member expression which ultimately leads to changes in gene expression. Our results support this hypothesis and demonstrate that osteoblasts are sensitive to changes in extracellular glucose and respond uniquely through an osmotic stress pathway involving protein kinase C and chronic induction of c-Jun expression and altered osteoblast phenotype.

3. Materials and Methods

3.1 Cell culture system

MC3T3-E1 cells (Sudo et al., 1983) were plated at 100,000 cells per 100 mM dish and fed every 2 days with alpha MEM (Gibco; Grand Island, NY) containing 5.5 mM glucose (normal level) and supplemented with 10% fetal calf serum. The typical concentration of insulin in the culture media is obtained from the serum component and ranges between 1 and 5 picomole concentration, a level lower than physiologic (50-800 pM), but consistent with early development of IDDM. Eleven days after plating and 24 hours after the last feeding, glucose or mannitol (0.5M stock) were added directly to the media in the tissue culture dish to yield the final concentrations of sugar noted for each experiment (7.5 to 22 mM). For kinase inhibition studies, 30 min prior to addition of sugar the cells were pre-treated with 50 nm of staurosporine or 200 nM of Ro-31-8220, concentrations lower than those previously reported to inhibit protein kinase C (Nose and Shibanuma, 1994; Beltman et al., 1996).

3.2 RNA analysis

Twenty four hours after feeding, glucose or mannitol (0.5 M stock) was added directly to the media in the tissue culture dish to yield a final monosaccharide concentration of 7.5 to 22 mM. Cells were washed and scraped in PBS at the indicated times following treatment. Cells were centrifuged at 800 X g for 5 minutes and quick-frozen in liquid nitrogen. Total RNA was extracted and analyzed by northern blot, as previously described (McCabe et al., 1995; Chomczynski P and N Sacchi, 1987).

Northern blots were hybridized with random primed (Random primed DNA labeling kit, Gibco) 32P-labeled complementary DNA probes specific for each AP-1 family member (generously provided by Dr. Rodrigo Bravo) and to cDNA probes for markers of osteoblast differentiation (McCabe et al., 1995 & 1996, Lian and Stein, 1992). Hybridzation signals were quantitated by phosphoimaging analysis and expressed relative to 18S ribosomal subunit levels.

3.3 Statistical analysis

All statistical analyses were performed using Microsoft excel data analysis program for t test analysis. Experiments were repeated at least three times unless otherwise stated. The autoradiographs shown are of one representative experiment. Values are expressed as a mean ± SEM except where indicated.

4. Results

4.1 Elevated levels of extracellular sugar influences osteoblast gene expression

The use of an osteoblast cell line, MC3T3-E1 cells, which exhibits gene expression in developmental manner similar to primary rat osteoblasts and bone *in vivo* (Quarles et al., 1992; Rodan et al., 1988), allowed us to examine modulation of one factor associated with diabetes, glucose, while leaving insulin levels constant. Previously we found that chronic treatment of osteoblasts with 16.5 mM glucose, a level seen in untreated diabetics, results in decreased mineralization (Zayzafoon et al., 1998). To determine if osteoblasts respond to acute glucose treatment, MC3T3-E1 cells were treated

(24 hours after feeding/media change) with 16.5 mM glucose which was directly added to the tissue culture medium. Cells were harvested at 1 and 24 hours after treatment. Within 24 hours of treatment gene expression was clearly modulated when compared to control cultures which have 5.5 mM glucose (normal *in vivo* and *in vitro* levels). Specifically, the level of collagen I mRNA was significantly increased by greater than two fold (Figure 3). In contrast, the level of osteocalcin mRNA was decreased to 30% of control levels. Osteopontin, actin, and histone H4 expression was unaffected (Figure 3). A two fold increase in collagen I expression was seen within one hour of treatment (Figure 4) demonstrating that collagen I expression is highly and rapidly responsive to elevation in extracellular glucose levels. In addition, the influence of extracellular glucose on collagen I expression was detectable with as little as 4.5 mM glucose addition (data not shown), a level that would be commonly seen in a diabetic patient.

4.2 Elevated extracellular sugar levels stimulate *c-jun* expression

Based on the role of AP-1 in immediate response of cells to extracellular stimuli (Morgan and Curran 1991; Angel and Karin 1991) and in the regulation of osteoblast growth, differentiation, and gene expression (McCabe et al., 1996) we next examined whether AP-1 family member expression in osteoblasts was modulated by glucose treatment. MC3T3-E1 cells were treated with 16.5 mM glucose for 1 hour. Northern blot analysis demonstrates a greater than 3 fold induction of *c-jun* expression (Figure 5), a rapid and transient induction in *c-fos* which is not always detectable by northern blot, and a small but not statistically significant induction of *fra-1* (data not shown). RNA levels of other AP-1 members (*jun B, jun D, fos B, fra-2*) did not change.

To examine the characteristics of the *c-jun* response a complete time course was performed with osteoblasts being harvested at 0, 0.5, 1, 3, 8, and 24 hours following the addition of 16.5 mM glucose. Figure 6 demonstrates that the induction of *c-jun* was maximal at 0.5-1 hour and was maintained even 24 hours after the addition of glucose. This response is unlike a serum response where *c-jun* levels rapidly decline after 1 hour (McCabe et al., 1995). Thus, glucose treatment leads to long term changes in transcription factor levels.

To determine if the effect on *c-jun* expression was concentration dependent osteoblasts were treated with 2, 4.5, 9.5, and 16.5 mM glucose (final concentration equal to 7.5, 10,15, and 22 mM glucose, respectively) and *c-jun* mRNA levels were examined 1 hour after treatment. Figure 7 demonstrates that as little as 4.5 mM of glucose added to osteoblasts is enough to stimulate *c-jun* mRNA levels. This level of glucose (10 mM final concentration; 180 mg/dL) as well as the addition of 9.5 mM glucose (15 mM final concentration; 270 mg/dL) is often seen in patients who are diagnosed with diabetes.

Although the levels of glucose used in this study are not normally used to study osmotic effects on cells, we still wanted to determine if any component of the osteoblast response results from osmotic stress. Therefore osteoblasts were also treated with mannitol, a non-absorbable sugar, to control for osmotic stress. Surprisingly, figures 3, 4, 5, 6 and 7 demonstrate that mannitol can completely mimic the effects seen with glucose. The influence of mannitol on *c-jun* was seen with as little as 4.5 mM addition. These results demonstrate that osmotic stress is the primary conveyor of the glucose-induced changes in gene expression shown in figures 3, 4, 5, 6 and 7.

4.3 Inhibition of protein kinase C blocks glucose induction of *c-jun* and collagen I expression

To begin to address the signaling pathways involved in the osteoblast response to glucose we treated MC3T3-E1 cells with glucose in the presence of a protein kinase C inhibitor, staurosporine. Northern blot analysis demonstrated that inhibition of protein kinase C blocked the induction of collagen I and *c-jun* expression after 1 hour of glucose treatment (Figure 8 A) while actin expression was not affected. Ro-31-8220, another inhibitor of protein kinase C, also suppressed the influence of glucose on *c-jun* and collagen I expression (Figure 8 B). Treatment with Ro-31-8220 alone had no effect on basal collagen I expression but did enhance *c-jun* expression as has previously been reported (Beltman et al., 1996). These findings further support an important role for protein kinase C in the osteoblast response to osmotic stress.

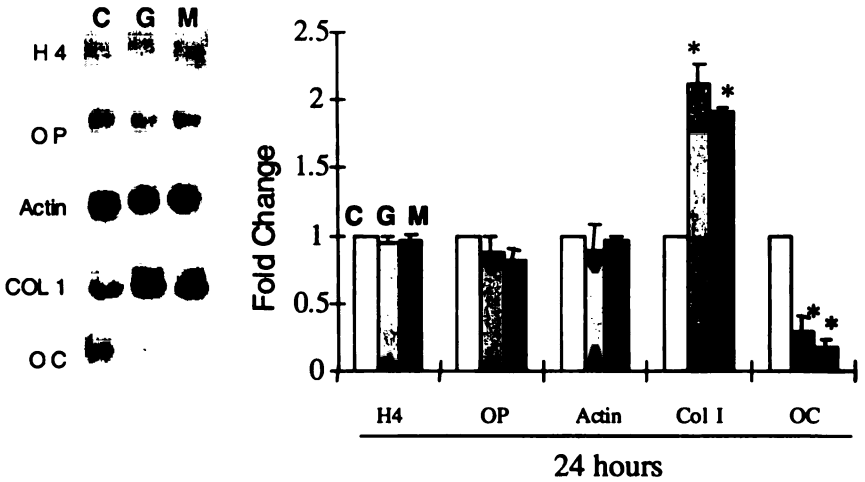


Figure 3. **Elevated levels of extracellular sugar influence osteoblast gene expression 24 hours after treatment.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. On day 11 (24 hours after the last feeding) glucose (G, 16.5 mM) or mannitol (M, 16.5 mM) were added directly to the tissue culture media of confluent cultures of osteoblasts to yield a final concentration of 22 mM monosaccharide. Control plates (C) were treated with PBS. Twenty-four hours after treatment the cells were harvested for RNA. Autoradiographs of northern blot membranes hybidized with cDNAs to histone H4 (H4), osteopontin (OP), actin, collagen I (COL I) and osteocalcin (OC) are shown. Levels of mRNAs (as determined by phosphoimager analysis) for control (white), 22 mM glucose (gray), or 22 mM mannitol (dark gray) treated cells were expressed relative to ribosomal 18S subunit RNA levels and are graphed as a fold increase relative to control levels set at 1. Values were obtained from 3-7 separate experiments and are expressed +/- SE. *$p<0.05$

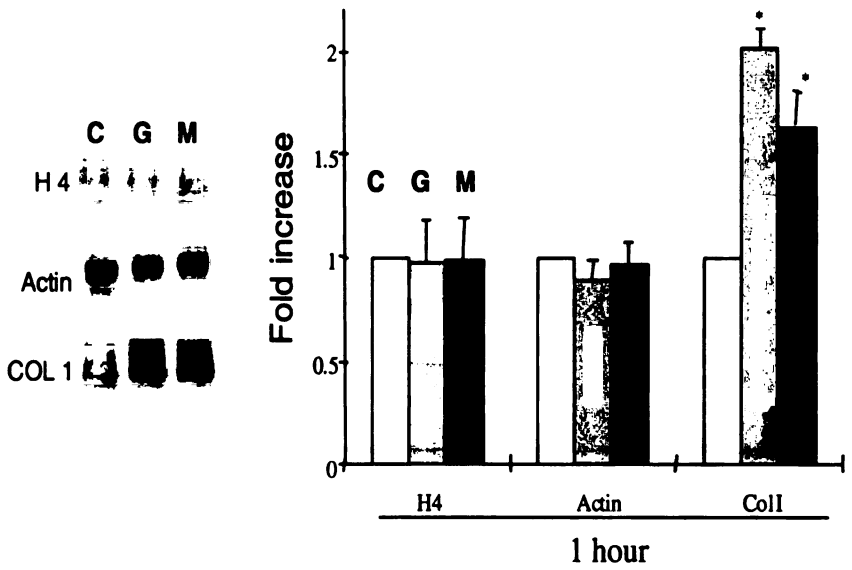


Figure 4. **Elevated levels of extracellular sugar influence osteoblast gene expression one hour after treatment.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. On day 11 (24 hours after feeding) glucose (G, 16.5 mM) or mannitol (M, 16.5 mM) were added directly to the tissue culture media of confluent cultures of osteoblasts to yield a final concentration of 22 mM monosaccharide. Control plates (C) were treated with PBS. One hour after treatment the cells were harvested for RNA. Autoradiographs of northern blot membranes hybidized with cDNAs to histone H4 (H4), actin, and collagen I (COL I) are shown. . Levels of mRNAs (as determined by phosphoimager analysis) for control (white), 22 mM glucose (gray), or 22 mM mannitol (dark gray) treated cells were expressed relative to ribosomal 18S subunit RNA levels and are graphed as a fold increase relative to control levels set at 1. Values were obtained from 3-7 separate experiments and are expressed +/- SE; *$p<0.05$

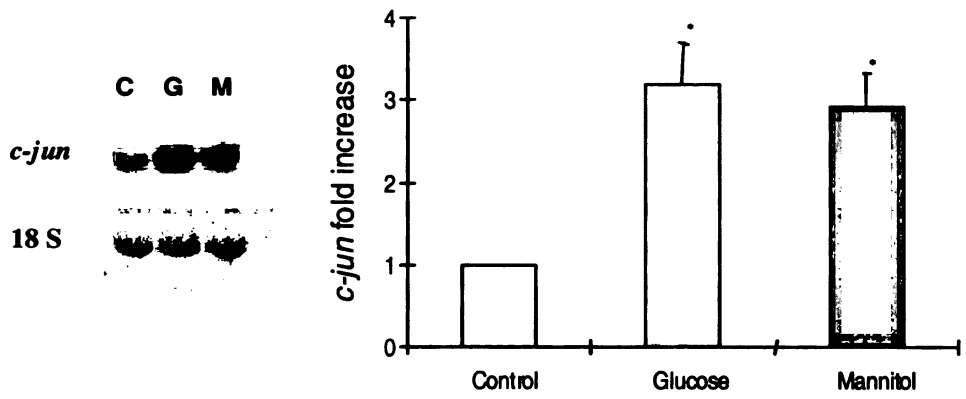
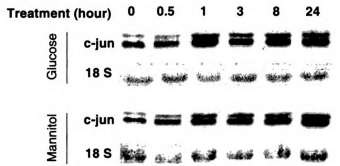


Figure 5. **Elevated extracellular sugar levels stimulate *c-jun* expression one hour after treatment.** On day 11 osteoblasts were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM. Cells were harvested 1 hour after addition of sugar for RNA extraction. The autoradiograph was obtained a northern blot hybridized to a cDNA specific for *c-jun*. The membrane contains RNA from control (C), 22 mM glucose (G), or 5.5 mM glucose + 16.5 mM mannitol (M) treated osteoblasts. Levels of *c-jun* mRNA (as determined by phosphoimager analysis) were expressed relative to ribosomal 18S subunit RNA levels and graphed relative to control values set at 1: control (white), 22 mM glucose (gray), or 22 mM mannitol (dark gray). Values were obtained from 7 separate experiments and are expressed +/- SE; *$p<0.01$.

Figure 6. **Glucose and mannitol treatment results in chronic elevation of *c-jun* expression.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. At day 11 osteoblasts were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM. Cells were harvested at 0, 0.5, 1, 3, 8, and 24 hours after the addition of sugar for RNA extraction. A) A representative autoradiograph containing RNA from glucose or mannitol treated osteoblasts hybridized to a cDNA specific for *c-jun* or 18 S ribosomal subunit RNA. B) Levels of *c-jun* mRNA (as determined by phosphoimager analysis) from glucose treated (gray) or mannitol treated (dark gray) osteoblasts were expressed relative to ribosomal 18S subunit RNA levels and graphed as a fold increase relative to basal control (0 hr) levels. Values were obtained from 3 separate experiments and are expressed +/- SE.

A.


Treatment (hour) 0 0.5 1 3 8 24
Glucose
c-jun
18 S
Mannitol
c-jun
18 S

B.

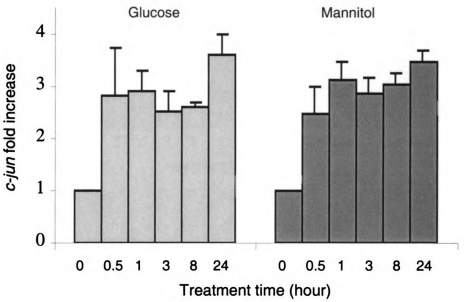
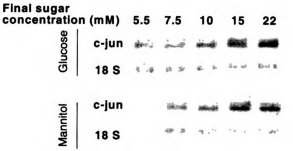

Glucose
Mannitol
c-jun fold increase
4
3
2
1
0
0 0.5 1 3 8 24
0 0.5 1 3 8 24
Treatment time (hour)

Figure 7. **Induction of *c-jun* by glucose or mannitol is concentration dependent.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. At day 11 (24 hours after feeding) osteoblasts were treated with additional glucose or mannitol to yield a final sugar concentration of 7.5, 10, 15, or 22 mM. Cells were harvested 1 hour after the addition of sugar and RNA extracted. A) A representative autoradiograph of a northern blot containing RNA from glucose or mannitol treated osteoblasts hybridized to a cDNA specific for *c-jun* or 18 S ribosomal subunit RNA. B) Levels of *c-jun* mRNA (as determined by phosphoimager analysis) from glucose treated (gray) or mannitol treated (dark gray) osteoblasts were expressed relative to ribosomal 18S subunit RNA levels and graphed as a fold increase relative to basal control (5.5 mM) levels. Values were obtained from 2 separate experiments.

A.


Final sugar concentration (mM)
5.5
7.5
10
15
22
Glucose
c-jun
18 S
Mannitol
c-jun
18 S

B.

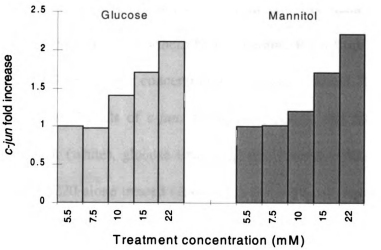
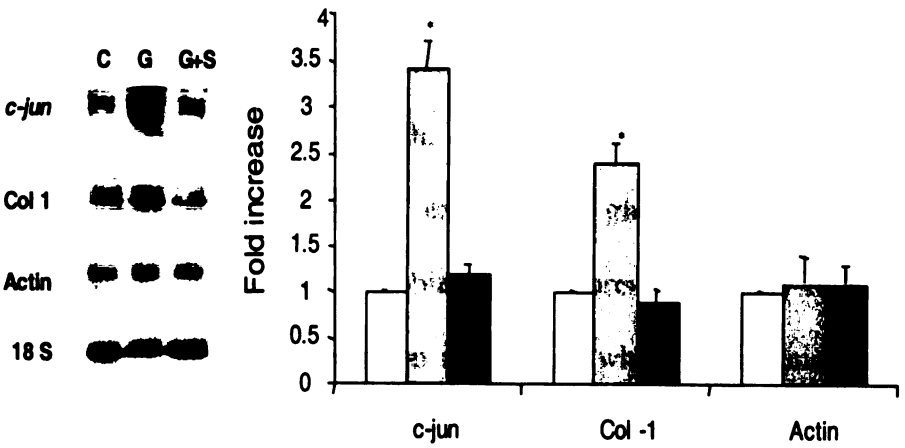
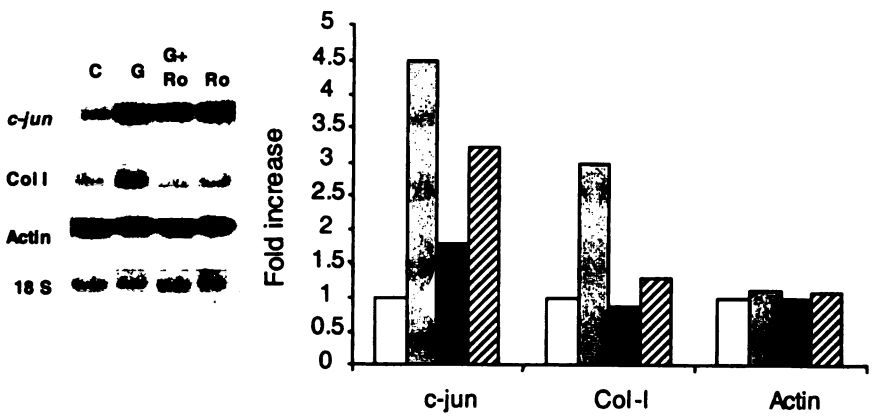

Glucose
Mannitol
c-jun fold increase
2.5
2
1.5
1
0.5
0
5.5
7.5
10
15
22
5.5
7.5
10
15
22
Treatment concentration (mM)

Figure 8. **Inhibition of protein kinase C blocks glucose induction of *c-jun* and collagen I expression.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. At day 11 (24 hours after feeding) osteoblasts were pretreated for 30 minutes with 50uM staurosporine (A) or with 100uM of Ro-31-8220 (B). Cells were then treated with 16.5 M glucose. After 1 hour cells were harvested and RNA extracted. A) Representative autoradiographs of a northern blot containing RNA from control (5.5 mM glucose), glucose (22 mM final sugar concentration) and glucose + staurosporine treated cells. Levels of *c-jun,* collagen I (COLI), and actin mRNA were quantitated (by phosphoimager analysis) from control (white), glucose treated (gray), or glucose + staurosporine treated (dark gray) cells. Values (+/- SE) were expressed relative to 18S rRNA levels and expressed relative to control levels set at 1; n=3, *$p<0.02$. Values for staurosporine alone treated cells did not differ from controls (data not shown). B) Representative autoradiographs of a northern blot containing RNA from control (5.5 mM sugar), glucose (22 mM final sugar concentration), glucose + Ro-31-8220, and Ro-31-8220 alone treated cells. Levels of *c-jun,* collagen I (COLI), and actin mRNA were quantitated from control (white), glucose treated (gray), glucose + Ro-31-8220 treated (dark gray), or Ro-31-8220 alone treated (diagonal lines) cells and expressed relative to control cells; n=2.

A.


C
G
G+S
c-jun
Col 1
Actin
18 S
Fold increase
4
3.5
3
2.5
2
1.5
1
0.5
0
c-jun
Col -1
Actin

B.


C
G
G+ Ro
Ro
c-jun
Col I
Actin
18 S
Fold increase
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
c-jun
Col-I
Actin

5. Discussion

Diabetes (IDDM) is marked by high glucose levels and is associated with bone loss, decreased osteoblast number, and decreased osteoid surface suggesting that osteoblast phenotype is altered. Few studies have examined the influence of increasing extracellular glucose on osteoblast growth, differentiation, and gene expression. Our results demonstrate that modulation of extracellular glucose concentration has an immediate and significant effect on osteoblast gene expression twenty-four hours after treatment: *c-jun* and collagen I expression is elevated while osteocalcin expression is decreased. Treatment with mannitol, a nonabsorbable sugar, completely mimics the effects of glucose hence the role of glucose metabolites can also be excluded and the role of osmotic stress can be concluded as a primary inducer of the effects seen on gene expression. This concept may be specific for osteoblasts which may be more sensitive to this form of stress than other types of cells. Osteoblast sensitivity to osmotic stress is demonstrated by the small addition of (4.5 – 16.5 mM) of either glucose or mannitol to osteoblasts which results in increase in *c-jun* expression and in collagen I expression. Elevation of extracellular glucose levels to 10-30 mM has been shown to influence gene expression in other cells including mesangial cells (Ingram et al., 1999), monocytes (Manduteanu et al., 1999) and fibroblasts (Benazzoug et al., 1998), potential mechanisms causing these changes are thought to stem from metabolic effects although effects on monocytes involve an osmotic component (Manduteanu et al., 1999).

Examination of glucose transporter expression by osteoblasts further supports a role for increasing extracellular glucose concentration in causing osmotic stress. It is known that osteoblasts do not express Glut 2 (the only high Km, 20mM, glucose

transporter; Waeber et al., 1994) but do express Glut 1 and Glut 3 glucose transporters (Thomas et al., 1996 a & b) which are known to have low Kms (1-2 mM). Thus, entry of glucose into the osteoblast is not significantly affected by increases in extracellular glucose (Zayzafoon and McCabe, Manuscript submitted). Therefore, an elevation in extracellular glucose should cause an osmotic stress which could be continuous or the osteoblast may adapt by generating osmolytes or modulating water and ion channel transport.

Increased expression of collagen I and other matrix mRNAs has been previously reported for other cell types exposed to glucose including mesangial cells (Ziyadeh et al., 1995) and fibroblasts (Han et al., 1999; Benazzoug et al., 1998). Consistent with our results, the promoter of collagen I is known to contain an functional AP-1 site (Chung et al., 1996). Yet an elevation in collagen I expression is somewhat contradictory to histological findings of decreased osteoid surface in diabetic animals (Verghaeghe et al., 1990; Hough et al., 1981). One possibility is that collagen protein synthesis, processing, or secretion is down regulated or altered by glucose treatment thereby negating the elevation in mRNA expression. In addition our results are focused on the specific modulation of extracellular glucose only, while *in vivo* findings may involve the additional effects of chronic exposure and the modulation of other factors including insulin, IGF-1, and amylin; changes in these and other factors could lead to a more complexly developed phenotype.

In contrast to the stimulation of collagen I expression, osteocalcin expression was suppressed by elevation in extracellular glucose. The osteocalcin promoter contains several AP-1 sites which have been demonstrated to be functional. c-Jun exhibits binding

activity at these sites and when overexpressed can suppress osteocalcin transcription (McCabe et al., 1996). This suppression is thought to result, at least in part, by the competition with osteocalcin inducing transcription factors (such as the vitamin D receptor) for overlapping promoter binding sites (Lian et al., 1991). Thus, suppression of osteocalcin expression in response to increased glucose is consistent with the elevation in *c-jun* expression which may be directly suppressing osteocalcin expression.

Activation of protein kinase C in diabetic tissue is a well-known outcome. It is hypothesized that metabolism of glucose, through the pentose phosphate shunt or through glycerolipid production, leads to an increase in diacylglycerol and subsequently protein kinase C activation. Our studies demonstrate that PKC is also involved in a nonmetabolic pathway involving osmotic stress. Specifically, induction of *c-jun* and collagen I mRNA levels by either glucose or mannitol can be blocked by inhibitors of protein kinase C, staurosporine and a more specific inhibitor Ro-31-8220.

Given that protein kinase C is a major activator of AP-1 expression and transactivation, it is not surprising that we found an elevation in *c-jun* expression in osteoblasts treated with increasing concentrations of extracellular glucose. In addition, the c-jun promoter contains an AP-1 site that allows for positive autoregulation. In mesangial cell cultures, Kreisberg et al. (1994) also report an increase in *c-jun* mRNA levels as well as c-fos mRNA levels at 0.5 and 2 hours after glucose treatment. Our time course studies clearly demonstrate a long-term induction in *c-jun* mRNA expression which is supported by an elevated c-jun signal at 24 hours in the study by Kreisberg et al. (1994). In two experiments we were also able to detect an induction of c-fos mRNA

expression at 0.5 hours after glucose treatment. However this increase was not found in subsequent experiments suggesting that it is highly rapid and transient.

Given that the expression pattern of c-Jun and collagen I is highest in proliferating osteoblasts while osteocalcin is a marker of mature osteoblasts, our results suggests that glucose treated osteoblasts may be "reverting" to a less differentiated state thereby decreasing mineralization and ultimately bone mass *in vivo*. Since both collagen I and osteocalcin promoters contain AP-1 sites which are respectively postively and negatively responsive to c-Jun, it is highly possible that induction of c-jun by glucose is involved in the effects on these markers of osteoblast phenotype.

Taken together our findings demonstrate that an additional effect of glucose, osmotic stress, should be considered to contribute to the complications of diabetes with regard to osteoblasts. Osteoblasts are highly sensitive to osmotic stress and respond through a protein kinase C dependent pathway that results in an increase in c-jun and collagen I expression and a decrease in osteocalcin expression. Future studies will determine the precise role of c-Jun and the signaling pathways involved in the changes in osteoblast gene expression.

IV. Osteoblast Osmotic Response to Elevated Extracellular Glucose *in vitro* and *in vivo* Involves AP-1

(Manuscript submitted to JBMR)

1. Abstract

Osteoblasts, the cells responsible for bone formation, express glucose transporters with a low K_m. In poorly controlled or untreated type I diabetes mellitus blood glucose levels are constantly high. Consequently, this condition should place the osteoblast under osmotic stress. Although controversial, several studies have demonstrated that diabetes type I is associated with decreased bone mass. Here we demonstrate that long-term culturing of MC3T3-E1 osteoblasts in elevated glucose or mannitol (final sugar concentration equal to 22 mM) results in increased alkaline phosphatase activity and decreased mineralization relative to euglycemic 5.5 mM cultures. Similar results were obtained *in vivo* following 7 daily mannitol injections over the calvaria.

Osteoblast growth was not affected by elevated glucose or mannitol treatment. Short-term responsiveness, as determined by upregulation of *c-jun* and collagen I mRNA levels was induced by a 1 hour treatment with 16.5 mM glucose, mannitol, or 3-O-methyl-glucose. In combination with osteoblast uptake of sugar not differing between 5.5 and 22 mM glucose, these results further support that osteoblasts are exhibiting an osmotic response. Similar acute responses were observed *in vivo*. To understand the role of AP-1 in the response A-FOS, a dominant negative AP-1 construct, was overexpressed in osteoblasts and shown to block endogenous *c-jun* and collagen I mRNA induction by elevated extracellular glucose. These findings demonstrate an important role for AP-1 in osteoblast osmotic responsiveness.

2. Introduction

Diabetic bone disease was recognized at the beginning of 20th century (Morrison and Bogan, 1927). IDDM is associated with osteoporosis (Auwerx et al., 1988; Krakauer et al., 1995), decreased bone mass (Hui et al., 1985; Levin et al., 1976) impaired skeletal development (Levin et al., 1976) and increased fracture rates (Bouillon, 1991; Meyer et al., 1993). A recent clinical study of patients with IDDM found that 67% of diabetic men and 57% of diabetic women suffered from osteopenia (bone mineral density >1 but < 2.5 standard deviation (SD) of the population) of the femoral neck and/or lumbar spine (Kemink et al., 2000). Osteoporosis (bone mineral density >2.5 SD) was diagnosed in 14% of the male patients. These effects are even more pronounced in diabetic rats (Verhaeghe et al., 1990). Diabetic rats also exhibit significantly less osseointegration of bone implants than control (McCracken et al., 2000). Histochemical studies demonstrate that osteoblasts are directly influenced by diabetes. Specifically, osteoblast number/surface is reduced (cuboidal osteoblasts are replaced by bone lining cells) and osteoid surfaces are markedly decreased with no reduction in osteoclast number (Verhaeghe et al., 1990; Sasaki et al., 1991). These changes do not correlate with bone loss resulting from low vitamin D or calcium levels, and they do not occur in semistarved animals (Shires et al., 1980).

Diabetes is diagnosed by an elevated blood glucose level that is greater than 200 mg/dl (11 mM) at any random testing (Emancipator, 1999). This level of glucose can cause significant changes in cellular uptake and metabolism of glucose in cells, such as those in the kidney, liver and beta islet cells of the pancreas that express GLUT2, a high Km (~ 20 mM) glucose transporter. On the other hand, osteoblasts, the cells responsible

for bone formation, express only GLUT1 and GLUT3 (Thomas et al., 1996 a; Thomas et al., 1996 b), glucose transporters with a low K_m, 1-2 mM and <1 mm, respectively (Bell, 1991). Therefore at euglycemic state, when glucose is 3-5.5 mM, these transporters have reached their maximal capacity to transport glucose. In poorly controlled or untreated type I diabetes mellitus glucose level is constantly high. Consequently, if the glucose treatment does not alter the number of glucose transporters on the osteoblast cell surface membrane, this condition should place the osteoblast under osmotic stress.

To form a bone tissue-like organization *in vitro* and *in vivo* osteoblasts undergo three stages of development: proliferation, extracellular matrix maturation, and extracellular matrix mineralization (Quarles et al., 1992; Owen et al., 1990). During extracellular matrix maturation alkaline phosphatase expression increases, while osteocalcin (marker of the final stage of differentiation and matrix mineralization) expression decreases. Regulation of gene expression during osteoblast differentiation occurs at the level of transcription although it is clear that post-translational modifications of transcription factors also play a critical role.

The AP-1 family of transcription factors (Fos and Jun proteins) are critical in the response of osteoblasts to changes in extracellular stimuli such as vitamin D (Candeliere et al., 1991), PTH (Clohisy et al., 1992; Lee et al., 1994; Koe et al., 1997; McCauley et al., 2001), IGF (Merriman et al., 1990), BMP-2, (Ohta et al., 1992), or TGF-ß (Breen et al., 1994; Machwate et al., 1995). All AP-1 family members contain a leucine zipper and a basic region required for dimer formation and DNA binding, respectively (Kouzarides and Ziff, 1988). Dimerization is a prerequisite for DNA binding at AP-1 response elements (5'-TGAg/cTCA-3'). AP-1 member levels as well as post-

translational modifications can influence DNA binding and transactivation (Gruda et al., 1994; Smeal et al., 1992). Modulation of Fos and Jun family member expression in bone by overexpression or targeted gene ablation further demonstrates a role for this family in the regulation of osteoblast growth and differentiation (McCabe et al., 1996; Jochum et al., 2001; Sabatakos et al., 2000; Grigoriadis et al., 1993; Sunters et al., 1998).

The influence of glucose on osteoblast phenotype has not fully been examined, especially with regard to changes in osteoblast gene expression and differentiation. Important mechanisms by which glucose can influence osteoblast differentiation include altering cell metabolism, extracellular osmolality, signal transduction pathways, and transcription factor activities. Previously, we have shown that elevation of extracellular glucose stimulates the expression of *c-jun* and collagen I, genes that contain AP-1 sites in their promoters (Zayzafoon et al., 2000). These findings suggest an important role for AP-1 in the osteoblast response to osmotic stress. Here we demonstrate that hyperosmotic stress does not alter osteoblast growth, but chronic treatment does suppress mineralization and is associated with increased alkaline phosphatase activity *in vitro* and *in vivo*. Furthermore, *in vitro* upregulation of *c-jun* and collagen I expression in response to osmotic stress is dependent upon AP-1 activity and also occurs *in vivo*.

3. Materials and Methods

3.1 Cell Culture System

MC3T3-E1 cells (Sudo et al., 1983), subcloned for maximal alkaline phosphatase staining and mineralization, were plated at 100,000 cells per 100 mM dish and fed every

2 days with alpha MEM (Gibco) containing 5.5 mM glucose (normal level) and supplemented with 10% fetal calf serum. Eleven days after plating and 24 hours after the last feeding, glucose, mannitol or 3-O-methylglucose (0.5M stock) were added directly to the media in the tissue culture dish to yield the final concentrations of sugar noted for each experiment (22 mM). For differentiation studies, upon confluency osteoblasts were fed with media containing 25 μg/ml ascorbic acid and 2 mM beta-glycerol phosphate (Wang et al., 1999).

3.2 Histology

Osteoblasts and dissected calvaria were fixed in 2% paraformaldhyde in phosphate buffered saline for 10 minutes and stored at 4°C in 0.1 M cacodlyic buffer as previously described (Breen et al., 1994). Alkaline phosphatase activity was visualized by incubating cell layers or calvaria for 30 minutes at 37 °C with 0.5 mg/ml naphthol AS-MX phosphate disodium salt and 2.8% (v/v) NN dimethyl formamide with 1 mg/ml Fast Red TR salt (Sigma Chemical Co., St Louis, MO) in a 0.1 M Tris-HCl buffer, pH 8.4. Mineral deposition was assessed by von Kossa staining of the cultures (30 minutes in 3% AgNO3) (Clark, 1981).

3.3 RNA Analysis

Twenty-four hours after feeding, glucose, mannitol or 3-O-methylglucose (0.5 M stock) was added directly to the media in the tissue culture dish to yield a final monosaccharide concentration of 22 mM. Cells were washed and scraped in PBS at the indicated times following treatment. Cells were centrifuged at 800 X g for 5 minutes and

quick-frozen in liquid nitrogen. Total RNA was extracted and analyzed by northern blot, as previously described in (McCabe et al., 1995; Chomczynski and Sacchi, 1987). Northern blots were hybridized with random primed (Random primed DNA labeling kit, Gibco) 32P-labeled cDNA probes specific for c-jun and Collagen 1 (Zayzafoon et al., 2000). Hybridization signals were quantitated by phosphoimaging analysis and expressed relative to 18S ribosomal subunit levels.

3.4 Glucose Media Measurement Assay

MC3T3-E1 cells were treated with 5.5 mM glucose for 11 days. Twenty four hours after the last feeding cells where treated with 16.5mM glucose or mannitol to yield a final sugar concentration of 22 mM. Sample 100μl was taken from the media at a different time point ranging from 0 to 24 hours. Glucose assay was done on the samples to determine the concentration of glucose according to manufacture protocol (Reagents for the quantitative determination of glucose, Sigma Diagnostics).

3.5 3-O-methylglucose Uptake Assay

The uptake of radiolabeled non-metabolized glucose analog [^{3}H] 3-O-methylglucose (specific activity of 60 Ci/mmole, Amersham Corp.) was measured as previously described (Asano et al., 1992). Specifically, twenty-four hours after feeding, glucose or mannitol (0.5 M stock) was added directly to the media in the tissue culture dish to yield a final monosaccharide concentration of 22 mM. At the indicated times and at 37°C, 0.4μCi or 1.6 μCi of [^{3}H] 3-O-methylglucose was added to the 5.5 mM or 22mM sugar treated plates, respectively, to maintain consistent 3-O-MG/sugar ratio. The glucose

uptake was halted after 10 seconds by adding 20 μM cytochalasin B followed by five washes with chilled 1x PBS. Cells were then lysed and radioactivity counted.

3.6 Cell Growth and Proliferation Assay

Cell proliferation was determined by direct cell counting of viable cells and by [^{3}H] thymidine incorporation assay. For direct cell counting, MC3T3-E1 cells were grown for different period of time as indicated in the study. Treatment duration varied from day 1 until day 4, 4-8, 8-12, 12-14 from plating. At the end of the treatment, the plates were washed 3 times with cold PBS and trypsinized for 1-3 minutes. Complete cell detachment from plates was confirmed by observation under microscope. Osteoblasts were resuspended in PBS and an aliquot was treated with 4% trypan blue. Viable cells which excluded the dye were counted using a hemocytometer.

For [^{3}H] thymidine incorporation, MC3T3-E1 cells were grown and treated as above. At the end of the treatment, [^{3}H] thymidine was added to the culture medium at concentration of 5 μCi per ml. Cells were then incubated for 30 minutes at 37°C. Culture medium was removed by aspiration and osteoblasts were washed twice with cold PBS. Plates were kept on ice and 10% cold trichloroacetic acid (TCA) was added for 5 minutes. Ten % SDS was added to cells for 2 minutes after the removal of TCA (McCabe et al., 1994).

3.7 In vivo analysis

Balb/c mice, 7 days old, were injected with 50 μl of PBS alone (vehicle) or vehicle containing 22 mM mannitol subcutaneously over the calvaria. This method has

been previously used successfully to examine osteoblast response *in vivo* to hormones such as PTH (McCauley et al., 2001), FGF (Dunstan et al., 1999) and TGF-β (Fujimoto et al., 1999). Calvaria were dissected and flash-frozen in liquid nitrogen one hour after injection. Samples were subsequently processed for RNA analysis as described above. For chronic studies, 7 day old Balb/c mice were injected daily as described above, for 7 days. Calvaria were dissected and processed for alkaline phosphatase and von Kassa staining as described above.

3.8 Transient transfection studies

Osteoblasts, MC3T3-E1 cells, were plated at a concentration of 100,000 cells per well of a 6 well dish. Twenty-four hours later cells were transfected with a luciferase reporter plasmid driven by four tandem copies of the AP-1 enhancer fused to TATA-like promoter (P_{TAL}) region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter (CLONTECH Laboratories); the same reporter vector without inserted promoter; or an SV40 promoter-beta galactosidase reporter. Five hours after transfection with the DNA-lipofectamine mix (Gibco), 1 mL of 20% FBS α-mem was added to the cells. Fifteen hours later, media was aspirated and replaced with fresh 10% FBS α-MEM. 24 hours later, cells were treated for 3 hours with the indicated treatment in the study and then harvested and lysed. Luciferase activity was read immediately using Promega luciferase assay system and a luminometer. For AP-1 dominant negative experiments, a CMV driven A-FOS expression plasmid (Olive et al., 1997) or an empty CMV expression vector were transfected into MC3T3-E1 cells as described above. Forty-eight

hours after transfection osteoblasts were treated with glucose, mannitol or PBS (control). One hour after treatment RNA was extracted and analyzed as described above.

3.9 Statistical analysis

All statistical analyses were performed using Microsoft excel data analysis program for t test analysis. Experiments were repeated at least three times unless otherwise stated. The autoradiographs shown are of one representative experiment. Values are expressed as a mean ± SEM except where indicated.

4. Results

4.1 Elevated levels of extracellular sugar suppress osteoblast mineralization both *in vitro* and *in vivo*

Previously, we have shown that acute elevation of extracellular glucose can alter MC3T3-E1 osteoblast gene expression. This suggests that long-term culturing of osteoblasts under high glucose conditions could have significant phenotypic effects. To test this, we fed osteoblasts every two days with media containing 5.5 mM glucose (control) or treated with an additional 16.5 mM glucose or mannitol for 21 days. Histochemistry demonstrated that staining of alkaline phosphatase activity, a marker of the matrix maturation stage of osteoblast differentiation, was higher in 22 mM glucose treated cultures compared to control (Figure 9). In contrast, mineralization as determined by Von Kossa staining was lower in the sugar-treated cultures. This result is consistent with the decreased bone formation seen clinically in diabetic patients (Kemink et al.,

2000). To determine if similar changes in osteoblast phenotype could be observed *in vivo*, 22 mM mannitol was injected subcutaneously over mouse calvaria daily for 7 days. Calvaria were processed for histology. Consistent with our *in vitro* findings, Figure 10 shows that mannitol treated calvaria have increased alkaline phosphatase activity and decreased mineralization compared to the saline injected controls. Similar results were obtained when glucose was injected (data not shown). Given that mannitol is not transported across the cell membrane nor is it metabolized, these findings suggest that osmotic stress suppresses late stages of osteoblast differentiation associated with mineralization, hence expression of genes associated with early stages of differentiation, alkaline phosphatase, is maintained.

4.2 Sugar treatment does not influence glucose uptake

To determine if the increase in extracellular glucose stimulates glucose uptake, a time course of 3-O-methyl-glucose uptake was examined in MC3T3-E1 cells. Osteoblasts cultured in 5.5 mM glucose for 10 days were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM sugar. Figure 11 demonstrates that elevation of extracellular glucose is not accompanied by an increase in glucose uptake. Correspondingly, measurements of glucose concentration in the medium over a twenty four-hour period after treatment also did not demonstrate a significant difference in glucose utilization among the various conditions (figure 12). These results are consistent with constitutive levels of low K_m glucose transporters present in the membranes of osteoblasts.

4.3 *c-jun* is induced within one hour in response to osmotic stress

Next, we treated osteoblasts with different sugars to further determine if absorption and/or metabolism are important for osteoblast responsiveness to increased extracellular sugar. We have previously shown that *c-jun* mRNA levels are increased in response to elevated extracellular glucose (Zayzafoon et al., 2000), therefore, we examined the induction of this gene as a marker of responsiveness. Osteoblasts were treated with 16.5 mM glucose (absorbed and metabolized), 3-O-methyl-glucose (absorbed but not metabolized), or mannitol (not absorbed or metabolized) to yield a final concentration of 22 mM. As shown in Figure 13, addition of any of the sugars resulted in a significant increase in *c-jun* mRNA levels within one hour of treatment. Thus, sugar absorption and metabolism are not required for osteoblast responsiveness to elevations in extracellular glucose. This finding confirms our glucose uptake assay results and demonstrates that the response is osmotic. It is also consistent with mannitol treatment causing phenotypic changes *in vivo* and *in vitro* that are similar to the changes seen *in vitro* with chronic glucose treatment.

4.4 Glucose treatment does not affect growth of MC3T3-E1 cells

Elevation of *c-jun* expression can be associated with growth so we next examined if increasing extracellular glucose was stimulating osteoblast proliferation. Osteoblasts were incubated in high glucose or mannitol containing media (addition of 16.5 mM sugar to obtain a final concentration of 22 mM sugar) for 4 days at different stages of the growth and differentiation time course and cell counts and thymidine incorporation were determined. As shown in Figure 14, elevation of extracellular sugar from day 1-4, 4-8, 8-

12, or 12-16 has no significant effect on osteoblast growth. Similarly, osteoblast number, as determined by cell counts, did not differ during and following these treatments. (data not shown).

4.5 Elevation of extracellular glucose increase AP-1 transactivation

Alternatively, induction of *c-jun* could influence the expression of other genes not associated with growth. For example, elevated extracellular glucose can increase expression of collagen I in osteoblasts as we have previously shown (Zayzafoon et al., 2000). To determine if AP-1 transactivation is indeed increased in response to elevations in extracellular glucose, osteoblasts were transfected with a luciferase reporter driven by four tandem copies of AP-1. We demonstrate in figure 15 that elevation of extracellular sugar increases AP-1 transactivation over 3 fold. We have previously shown that PKC is important for the *c-jun* induction (Zayzafoon et al., 2000), therefore, it is not surprising to find an increase in AP-1 transactivation as it is known that an increase in AP-1 transactivation is associated with the activation of PKC (Angel and Karin, 1991).

4.6 AP-1 dominant negative, A-FOS, blocks *c-jun* and Collagen I Induction

The promoter of collagen I contains a functional AP-1 site (Katai et al., 1992) and therefore could be responsive to increased *c-jun* expression. To determine the importance of AP-1 in the induction of collagen I we utilized an AP-1 dominant negative construct, A-FOS (Olive et al., 1997), to inhibit AP-1 binding activity. A-FOS has its basic region replaced by an acidic domain; this modified domain binds to the basic region of its Jun partner and therefore inhibits the AP-1 complex from binding DNA. Compared to vector

transfected cells, A-FOS suppressed the glucose induced increase in endogenous collagen I mRNA levels (Figure 16) consistent with collagen I being a downstream target of c-Jun. Interestingly, A-FOS also suppressed induction of *c-jun* by elevated extracellular glucose. This is consistent with the positive autoregulatory AP-1 site located within the c-jun promoter (Angel et al., 1988). Thus, AP-1 binding and transactivation are important in the osmotic upregulation of both *c-jun* and collagen I expression.

4.7 *c-jun* and COL I are induced within one hour in response to elevated extracellular mannitol *in vivo*

To examine acute responsiveness *in vivo*, mice were given a single injection of 22 mM mannitol subcutaneously over the calvaria. One hour after injection RNA was extracted from mannitol treated and saline injected controls. Figure 17 shows that both *c-jun* and collagen I mRNA expression are induced by the mannitol injection while actin expression is not affected. These results are again consistent with our *in vitro* response data.

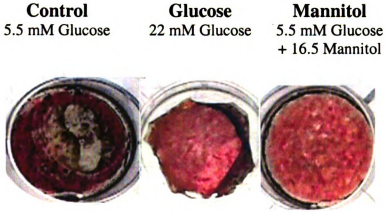


Figure 9. **Elevated levels of extracellular sugar suppress osteoblast mineralization.** MC3T3-E1 cells were fed regularly with media containing 5.5 mM glucose (control, left), 22 mM glucose (glucose treated, center) and 5.5 mM glucose +16.5 mM mannitol (mannitol treated, right). Cells were treated beginning on day 4 and were fixed on day 28. Following fixation, samples were stained for alkaline phosphatase activity (red) and mineralization (Black). Similar results were seen in three other experiments.

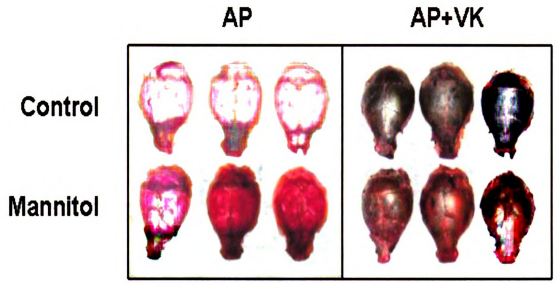


Figure 10. **Osmotic stress suppresses bone mineralization in mice.** Mice 7 days old were inject subcutaneously with 22 mM mannitol over the calvaria for 7 days. Calvaria were then harvested and fixed. The specimens were then stained for alkaline phosphatase activity (AP, red) and mineralization (VK, black).

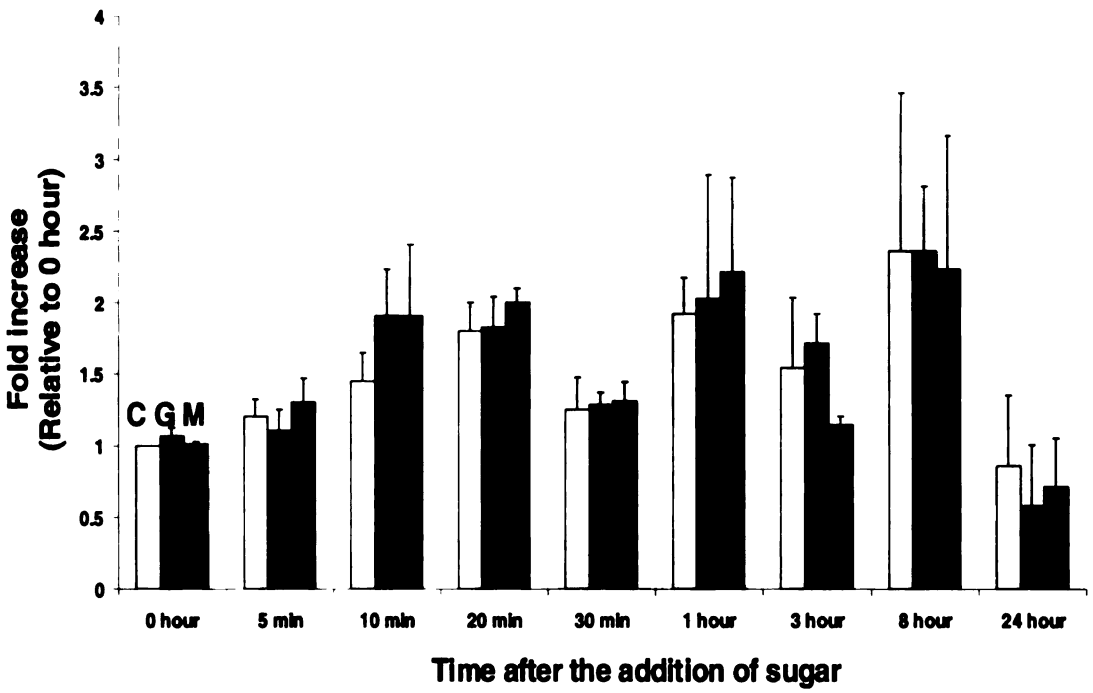


Figure 11. **Sugar treatment does not influence glucose uptake.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. On day 11 (24 hours after feeding) glucose (G, 16.5 mM) or mannitol (M, 16.5 mM) were added directly to the tissue culture media of confluent cultures of osteoblasts to yield a final concentration of 22 mM monosaccharide. At the indicated times [^{3}H] 3-O-methylglucose a non-metabolized glucose analog was added. Glucose uptake was halted after 10 seconds by adding 20 μM cytochalasin B followed by five washes with chilled 1x PBS. Cells were then lysed and radioactivity counted. Data are the mean ± SD and are combined from two experiments, triplicate determinations for each condition.

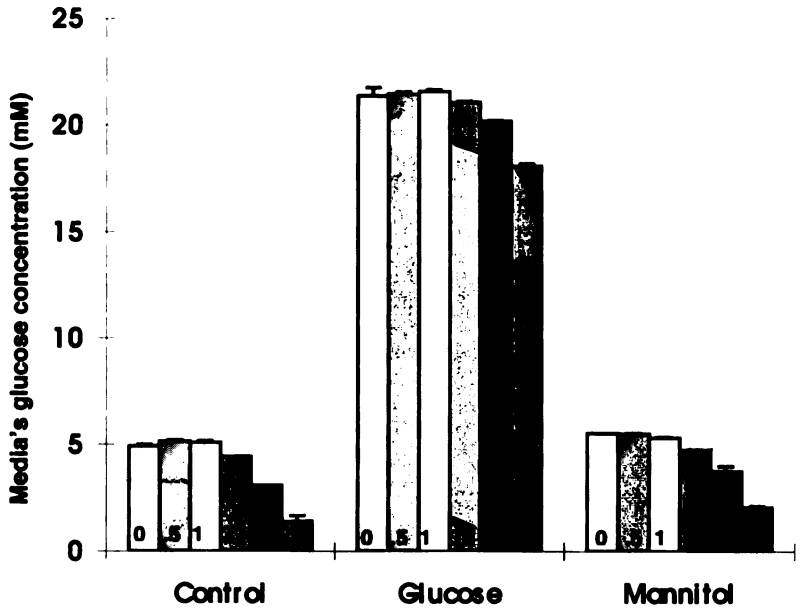


Figure 12. **Steady influx of glucose into MC3T3-E1 cells under hyperosmolar conditions.** MC3T3-E1 cells were treated with 5.5 mM glucose for 11 days. At day 12, 16.5mM glucose or mannitol was added to the media. 100µl sample was taken from the media at a different time point ranging from 0 to 24 hours. Glucose assay was done on the samples to determine the concentration of glucose. Data are the mean ± SD and are combined from two experiments, triplicate determinations for each condition.

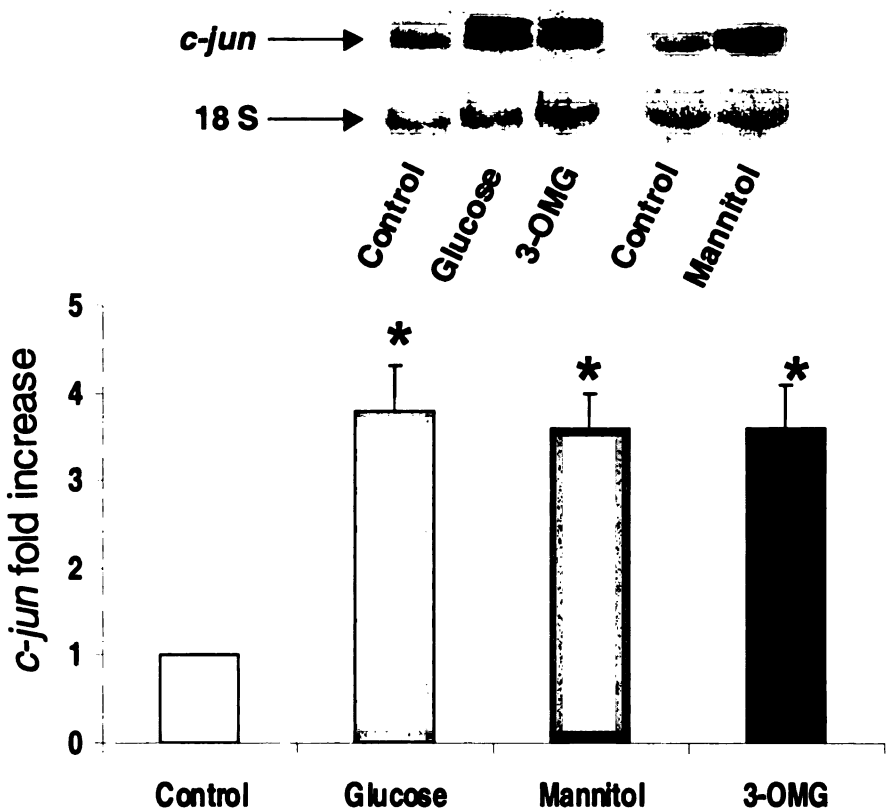


Figure 13. ***c-jun* is induced within one hour in response to elevated extracellular sugar.** MC3T3-E1 cells were cultured in 5.5 mM glucose. On day 12 osteoblasts were treated with glucose, mannitol or 3-O-Methyl-D-Glucose (3-OMG) to yield a final concentration of 22 mM (actual levels were 5.5 mM glucose and 16.5 mM sugar). Sugars were added directly to the media (24 hours after feeding) to prevent any influences from changing the media. *c-jun* expression was analyzed by northern blot analysis using RNA extracted from cells harvested at one hour after the addition of sugar to the media. Levels of *c-jun* mRNA (as determined by phosphoimager analysis) were expressed relative to ribosomal 18S subunit RNA levels and plotted relative to control values set at 1. Values were obtained from 3 separate experiments and are expressed +/- SE; *$p<0.05$.

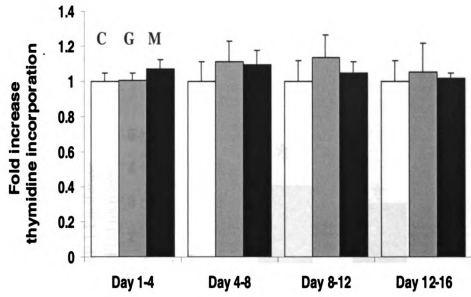


Figure 14. **Glucose treatment does not affect growth of MC3T3-E1 cells.** MC3T3-E1 cells were treated with 5.5 mM glucose (C), 16.5 mM glucose (G), or 16.5 mM mannitol (M) to yield a final concentration of 22 mM monosaccharide. Treatment duration was for 4 days and began on day 1,4,8 or 12 after plating. At the end of the treatment duration, 5 μCi/mL of [^{3}H] thymidine was added for 30 minutes and thymidine incorporation was measured. Data are the mean ± SEM and are combined from three experiments, triplicate determinations for each condition.

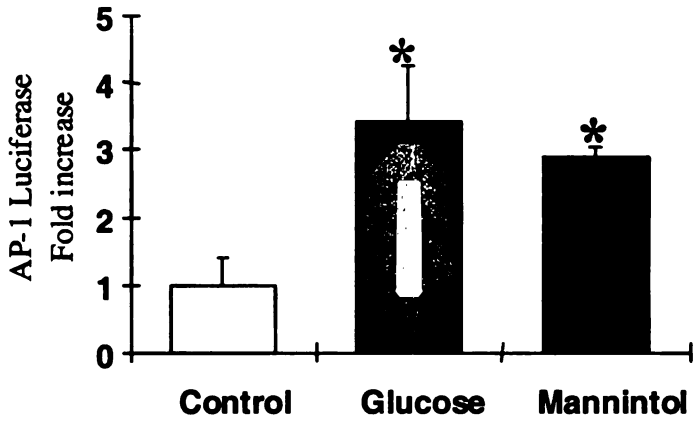


Figure 15. **Elevation in extracellular sugar increases AP-1 transactivation.** MC3T3-E1 cells were transfected with luciferase reporter plasmid driven by four tandem copies of the AP-1. Forty-eight hours later, osteoblasts were treated for 3 hours with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM of monosaccharide. Cells were then harvested and lysed for luciferase activity detection. Data are the mean ± SE and are combined from four experiments. Data are the mean ± SEM and are combined from three experiments, triplicate determinations for each condition. *$p<0.05$.

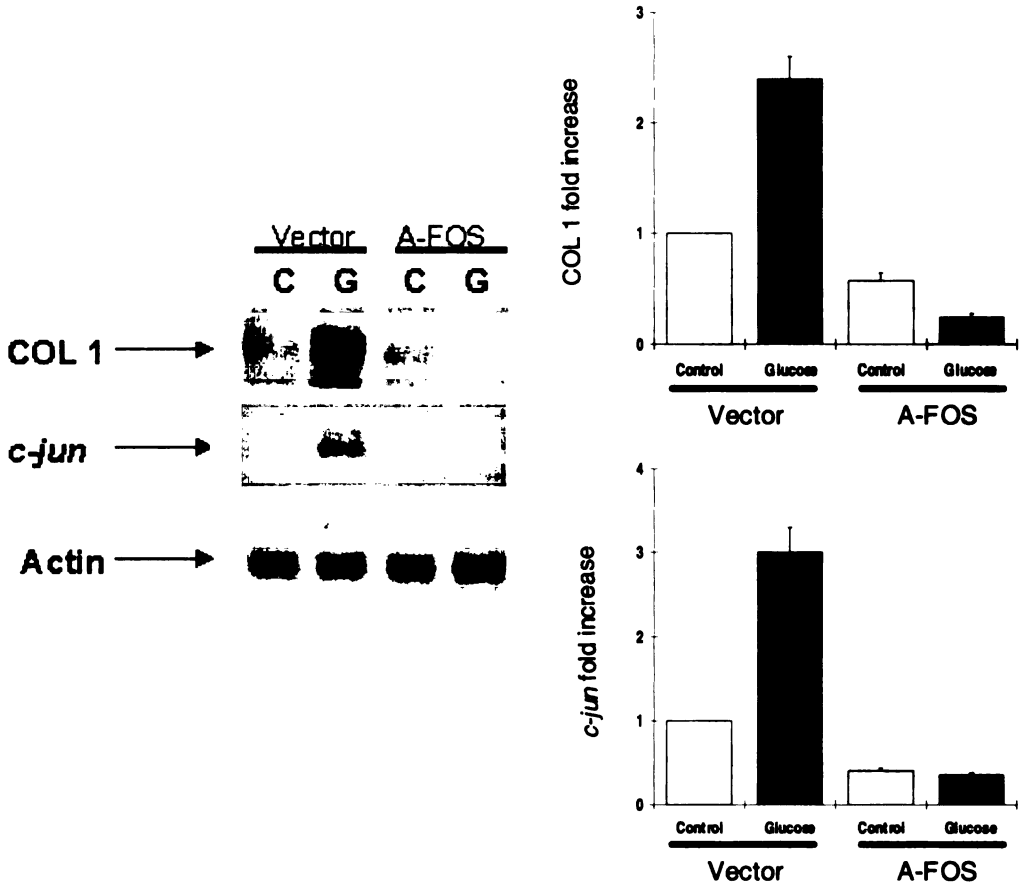


Figure 16. **AP-1 dominant negative, A-FOS, blocks *c-jun* and Collagen 1 Induction.** MC3T3-E1 cells were transfected with CMV vector alone or CMV A-FOS expression plasmid. Cells were treated with 16 mM glucose to yield a final concentration of 22 mM of monosaccharide. One hour later cells were harvested for RNA extraction and *c-jun* and COL 1 expression measured by northern blot analysis, representative autoradiographs are shown. Data are the mean ± SD and are combined from two experiments.

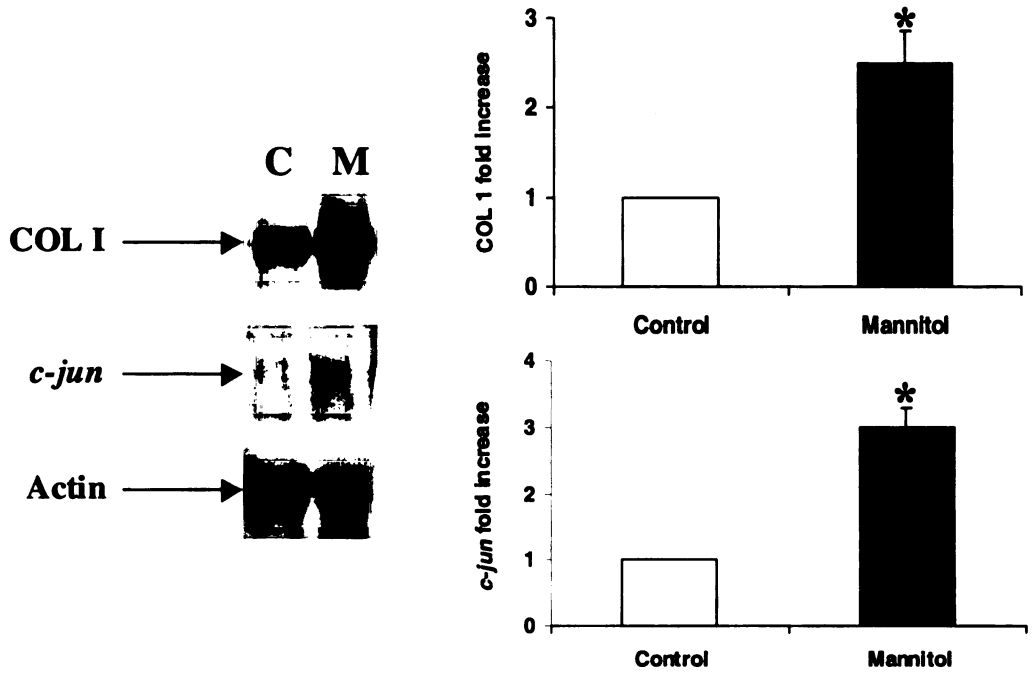


Figure 17. **Osmotic stress *in vivo* induces *c-jun* and COL I within one hour.** Balb/c mice, 7 days old, were injected with 50 ul of PBS alone (vehicle) or vehicle containing 22 mM mannitol subcutaneously over the calvaria. Calvaria were dissected and flash-frozen in liquid nitrogen one hour after injection. *c-Jun* and COL 1 expression was analyzed by northern blot analysis using RNA extracted from specimens. Levels of *c-jun* mRNA (as determined by phosphoimager analysis) were expressed relative to ribosomal 18S subunit RNA levels and graphed relative to control values set at 1. Values were obtained from 3 separate experiments and are expressed +/- SE; *$p<0.05$.

5. Discussion

Elevated blood glucose levels greater than 200 mg/dl (11 mM) are the diagnostic measure for diabetes. Elevations in extracellular glucose have been shown to influence a variety of tissues including vascular, kidney, and hematopoietic cells. Here we show that osteoblasts are also influenced by this condition. Long-term treatment of MC3T3-E1 osteoblasts with 22 mM glucose (a physiologically relevant hyperosmolarity) results in decreased mineralization. This is consistent with clinical data that suggest diabetes type I is associated with decreased bone mass in humans (Auwerx et al., 1988; Krakauer et al., 1995; Hui et al., 1985; Kemink et al., 2000) as well as in rodent models (Verhaeghe et al., 1990; McCracken et al., 2000) and histochemical studies that suggest osteoblast differentiation is suppressed (Verhaeghe et al., 1990). Mannitol treatment reproduced results seen with glucose treatment demonstrating that osmotic stress is involved in the response. We did notice some slight differences between mannitol and glucose treatments such as mannitol being a better inhibitor of mineralization. This may stem from mannitol being a purely osmotic effector. In addition, longterm treatment with elevated glucose could have some metabolic effects that we did not detect in our short-term uptake studies. Our chronic calvarial injections further demonstrate that the osmotic response we are seeing is not simply an artifact of the *in vitro* culture system or of our MC3T3-E1 cell line. Specifically, daily subcutaneous injections of 22 mM mannitol or 22 mM glucose over the calvaria suppress mineralization. This is the first *in vivo* evidence that osteoblast differentiation can be suppressed by osmotic stress. Interestingly, both *in vitro* and *in vivo* systems exhibited increased alkaline phosphatase expression in response to elevated extracellular glucose treatment. This finding could

result from the progression of osteoblast differentiation being "blocked" in the matrix maturation stage, marked by alkaline phosphatase expression (Owen et al., 1990), and therefore osteoblasts are inhibited from entering the final stage of differentiation associated with mineralization. This is consistent with our findings of suppressed osteocalcin expression, a marker of fully differentiated osteoblasts, in response to glucose treatment (Zayzafoon et al., 2000). Recently, Balint et al. have examined MC3T3-E1 phenotype in response to elevated glucose treatment (Balint et al., 2001). After 30 days of treatment alkaline phosphatase activity was increased while calcium deposition was decreased but nodule size and number was increased. Unlike our studies these changes were not seen in mannitoltreated cultures. Differences between findings may result from different MC3T3-E1 cell populations (Wang et al., 1999), as well as differences in the time of analysis. Osteoblasts cultured for 30 days can exhibit different metabolic needs, such as higher glucose utilization, and different responses compared to less confluent and less differentiated cultures. In our studies, cells were fed daily after day 10 to try to accurately maintain the level of glucose in the media.

Although differentiation was suppressed, we did not observe a change in osteoblast growth in response to elevated glucose. We examined growth following 4 day periods of glucose (22 mM) treatment (day 1-4, 4-8, 8-12, 12-16) to access if osteoblasts at a particular stage of differentiation could be influenced to grow by elevated extracellular glucose levels. In contrast, (Terada et al., 1998) found that osteoblast (MG-63 cells) IGF-1 induced growth was decreased by elevated extracellular glucose whereas Balint et al. (2001) found that glucose alone stimulated day 30 osteoblast growth. Differences between these studies include sugar concentration, time point of analysis, and

cell types. For example, growth seen in day 30 osteoblast could result from an elevated carbohydrate requirement of osteoblasts at this stage.

The characteristics of an osteoblast suggest that it should responds to elevated extracellular sugar through an osmotic response. Principally, osteoblasts express two glucose transporters GLUT1 and GLUT 3 (Thomas et al., 1996 a; Thomas et al., 1996 b). Each has a relatively low K_m, 1-2 mM and <1 mm, respectively (Bell, 1991). Therefore at euglycemic state, when glucose is 3-5.5 mM, these transporters have reached their maximal capacity to transport glucose. When extracellular glucose levels are increased it cannot enter the cell and be metabolized but rather it puts osteoblasts under osmotic stress. This is supported by our finding that glucose uptake is not modulated by elevating extracellular glucose levels. Similarly, mesangial and endothelial cells that also express GLUT 1 and GLUT 3 and not GLUT 2 have also been shown to exhibit an osmotic response to small elevations in extracellular glucose (Kreisberg et al., 1994; Duzgun et al., 2000). To definitively test this issue in osteoblasts we examine acute stimulation of *c-jun* and collagen I expression in response to elevated glucose, mannitol (not absorbed), or 3-O-methyl glucose (absorbed but not metabolized). Consistent with our previous studies (Zayzafoon et al., 2000) all sugars stimulated *c-jun* and collagen I expression demonstrating that indeed absorption and metabolism are not required for the response.

Small changes in extracellular osmolarity have been shown to influence other cells. For example (Park et al., 2000) demonstrated that high glucose can induce intercellular adhesion molecule-1 (ICAM-1) expression through an osmotic effect in rat mesangial cells. Monocytes as well elicited an osmotic response due to elevation in extracellular glucose involving ICAM-1 (Manduteanu et al., 1999). When fibroblasts

were cultured in physiologically relevant hyperosmolar medium (10-15 mM glucose) both mRNA and protein levels of collagen III were increased. Fibronectin was also increased (Benazzoug et al., 1998). These findings demonstrate that a moderate increase in extracellular glucose can have a dramatic effect on gene expression in a variety of cells including osteoblasts.

The induction of *c-jun* by osmotic stress is consistent with its role as an immediate response gene and its activity being regulated by MAP kinase signaling pathways that have been shown to be responsive to osmotic stress (Rosette and Karin, 1996; Duzgun et al., 2000; Sheikh-Hamad et al., 1998). Similar to our findings (Kreisberg et al., 1994) demonstrated that *c-jun* was induced by 15 mM glucose in mesangial cells. Upregulation of collagen I is consistent with it being an AP-1 responsive gene (Katai et al., 1992). Increases in extracellular matrix have been described in other tissues in response to diabetes. Collagen I and other matrix mRNAs have been previously reported to be elevated in response to extracellular glucose in mesangial cells (Ziyadeh et al., 1995) and fibroblasts (Han et al., 1999; Benazzoug et al., 1998). However an upregulation in osteoblast collagen I expression is not consistent with reports of decreased osteoid surface in diabetic bone (Verhaeghe et al., 1990). One possibility is that collagen RNA levels may not be directly related to matrix levels as a result of down regulation of synthesis, processing or secretion. On the other hand some type of feed back system may exist where collagen I expression is increased but it's degradation is increased. Collagenase-3 expression is also responsive to AP-1 levels (Hess et al., 2001; Winchester et al., 2000; Varghese et al., 2000; Rydziel et al., 2000) and could mask any increase in collagen I matrix production.

Here we also demonstrate that our acute and chronic *in vitro* results are consistent with the *in vivo* responses and are not an artifact of the tissue culture system. Subcutaneous injections above the calvaria have been previously used to examine osteoblast response *in vivo* to hormones such as PTH (McCauley et al., 2001), FGF (Dunstan et al., 1999) and TGF-β (Fujimoto et al., 1999).

The use of a dominant negative AP-1 construct, A-FOS demonstrates that osmotic induction of collagen I and *c-jun* mRNA is dependent upon AP-1 activation. These findings are consistent with both gene promoters containing functional AP-1 sites (Olive et al., 1997). The AP-1 site within the *c-jun* promoter has been shown to be involved in the amplification of c-Jun levels (Angel et al., 1988).

V. p38 and ATF-2 involvement in osteoblast osmotic response to elevated extracellular glucose

1. Abstract

Poorly controlled or untreated type I diabetes mellitus is characterized by hyperglycemia and is associated with decreased bone mass and increased fracture rates. Cells such as osteoblasts that lack the high K_m glucose transporter (Glut2) have limited capability to absorb glucose and consequently are placed under hyperosmolar stress. Investigation of osmotic stress in mammalian tissues has focused principally on kidney and cellular responses to hyperosmolar conditions above 600 mOsm. Recently, we have shown that osteoblasts are sensitive to osmotic stress and respond to changes in extracellular glucose and mannitol as little as 5 to 10 mM (180-270 mg/dL). The osteoblast osmotic response is dependent upon protein kinase C activity and results in upregulation of *c-jun* and modulation of collagen I and osteocalcin expression. To determine if diabetic hyperosmolar stress plays a role in activating MAP kinases in osteoblasts, we treated MC3T3-E1 osteoblasts (grown in 5.5 mM glucose) with 16 mM glucose or mannitol for one hour. Immunoblots of phosphorylated p38 demonstrate significant activation of p38 MAP kinase by both glucose and mannitol and no change in p38 levels. Increased activity of p38 MAP kinase was not inhibited by staurosporine, suggesting a PKC-independent pathway in this activation. This increase was time dependent peaking at 20 minutes and staying detectable after 24 hours. It was also concentration dependent, being detectable at concentration as low as 10 mM and increasing gradually as total sugar concentration increased up to 22 mM. ATF-2

activation followed the same pattern as phospho p38. EMSA studies showed an increase in CRE DNA binding 1 hour after hypertonic treatment with glucose or mannitol. Supershift analysis demonstrated the involvement of ATF-2 in this binding. Corresponding to EMSA results, CRE transactivation increased 3 hours after sugar treatment. SB 203580, a p38 MAP kinase inhibitor, inhibited ATF-2 phosphorylation as well as CRE transactivation. Therefore, we propose that increased p38 MAP kinase activity and ATF-2 phosphorylation contribute to CRE activation. These findings suggest that osmotic stress modulates osteoblast signaling pathways contributing to at least one complication of diabetes, osteoporosis.

2. Introduction

Diabetes is diagnosed by an elevated blood glucose level that is greater than 200 mg/dl (11 mM) at any random testing (Emancipator, 1999). In poorly controlled or untreated type I diabetes mellitus glucose level is constantly high. Consequently, this places the osteoblasts under osmotic stress. Response to elevated extracellular glucose differs greatly between cells. We have shown previously that elevation of extracellular sugar elicits an osmotic response in osteoblasts (Zayzafoon et al., 2000; Zayzafoon and McCabe, manuscript submitted).

Cells respond to different extracellular inputs from their environment by activation of protein kinase cascades. These complex networks allow amplification of the signal and can mediate the required diversity of cellular responses. Environmental cues direct osteoblasts to proliferate and differentiate and MAP kinase pathways provide a key link between the membrane bound receptors that receive these cues and changes in the

pattern of gene expression. The MAP kinase signaling pathways have been implicated in playing a role in mediating the osmotic stress adaptive mechanisms (Roger et al., 1999; Sheikh-Hamad et al., 1998; Rizoli et al., 1999; Rosette and Karin, 1996). The first adaptive process occurring in response to extracellular hypertonicity-induced cell shrinkage is a regulatory cell volume increase (RVI) which results from the stimulation of ion transporters (Lytle and Forbush, 1996; Grinstein et al., 1986) and reviewed extensively in (Parker, 1993). A second adaptive mechanism, in mammalian cells, is the induction of genes encoding proteins involved in the accumulation of intracellular "compatible osmolytes' within hours and days (Yamauchi et al., 1992; Kwon et al., 1992; Uchida et al., 1992; Bohren et al., 1989; Garcia-Perez et al., 1989). To date, the effects of MAP kinases have been mostly attributed to the control of gene transcription via phosphorylation of nuclear transcription. This eventually leads to gene modulation, which plays part in the cellular response to osmotic stress (Hoffert et al., 2000; Ishida et al., 1999; Schaffler et al., 2000).

IDDM is associated with an extensive list of late complications involving nearly every tissue. One major concern to young and aging diabetics is the association of IDDM with osteoporosis, decreased bone mass, impaired skeletal development and increased fracture rates (Bouillon, 1991; Auwerx et al., 1988; Krakauer et al., 1995; Hui et al., 1985; Levin et al., 1976; Meyer et al., 1993). Diabetic bone disease was first recognized at the beginning of the twentieth century (Morrison and Bogan, 1927) and described as retardation of bone development and bone atrophy in children with long-standing diabetes. A recent clinical study (Kemink et al., 2000) showed a high prevalence of osteopenia in patients with IDDM. It showed that 67% of the diabetic men and 57% of

the diabetic women suffered from osteopenia of the femoral neck and/or lumbar spine. These effects are even more pronounced in diabetic rats. Verhaeghe et al., (1990) showed that long standing diabetes in BB rats results in severe low-turnover osteoporosis probably related to decreased osteoblasts recruitment and/or function.

Previously, we have shown that elevation of extracellular glucose stimulates the expression of *c-jun* and collagen I, genes that contain AP-1 sites in their promoters (Zayzafoon et al., 2000). The use of PKC inhibitors as well as an AP-1 dominant negative (A-Fos) abolish this induction (Zayzafoon et al., 2000; Zayzafoon and McCabe, manuscript submitted). Morooka et al., (1995) demonstrated that ATF-2 and c-Jun DNA binding activity is highly regulated in response to stress. This complex then targets various promoter sites, including the cAMP response element, CRE, (Cai et al., 2000). Here we demonstrate that hyperosmotic stress caused by a physiologically relevant increase in extracellular glucose results in the activation of p38 MAP kinase and its downstream transcription factor, ATF-2. This activation is associated with an increase in CRE DNA binding and its transactivation. SB 203580, p38 MAP kinase inhibitor, was able to inhibit the phosphorylation of ATF-2 as well as CRE transactivation. These results demonstrate that p38 MAP kinase and ATF-2 are involved in the osteoblast response to moderate osmotic stress.

3. Materials and Methods

3.1 Cell Culture System

MC3T3-E1 cells (Sudo et al., 1983) were plated at 100,000 cells per 100 mM dish and fed every 2 days with alpha MEM (Gibco; Grand Island, NY) containing 5.5 mM glucose (normal level) and supplemented with 10% fetal calf serum. The typical concentration of insulin in the culture media is obtained from the serum component and ranges between 1 and 5 picomole concentration, a level lower than physiologic (50-800 pM), but consistent with early development of IDDM. Eleven days after plating and 24 hours after the last feeding, glucose or mannitol (0.5M stock) were added directly to the media in the tissue culture dish to yield the final concentrations of sugar noted for each experiment (7.5 to 22 mM). For kinase inhibition studies, 30 min prior to addition of sugar the cells were pre-treated with 10μM of SB 203580, concentrations previously reported for the inhibition of p38 activity (Nadkarni et al., 1999; Volonte et al., 2001).

3.2 Whole cell protein extraction

MC3T3-E1 (1 100mm plate) were harvested for whole cell protein preparation. Cells were washed with chilled 1 x PBS and left on ice 3 minutes. Cells were then harvested and centrifuged at 800 X g for 5 minutes at 4C. Lysis buffer containing 50mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 1%Triton X100 and 10% Glycerol was used to lyse cells and extract protein. A cocktail of protease and phosphatase inhibitors was added to the lysis buffer (1mM sodium ortho vanidate, 2 mM PMSF, 5 μg/ml aprotinin, 1mM EGTA, 10 mM NaF, 1 mM Na pyrophosphate and 0.1 mM βglycerolphosphate).

Cells are then centrifuged at 14,000 rpm for 30 minutes at 4C. Protein concentration from the supernatant was quantitated by the Biorad DC protein detection system.

3.3 Nuclear extract isolation

Osteoblasts (1 100mm plate) were harvested for nuclear extract preparation as previously described (McCabe et al., 1996). Briefly, nuclei were isolated by detergent lysis of the cells. Nuclei are then treated with a hypotonic solution followed by a 30-minute incubation at 4C in a high salt (600 mM KCl) solution. Nuclei are then centrifuged at 14,000 rpm and the supernatant represents the nuclear extract. All solutions in this procedure contained a cocktail of protease inhibitors including: PMSF (5mM), pepstatin (1μg/ml), TPCK (70μg/ml), leupeptin (0.5μg/ml), spermidine (1mM) and spermine (1mM) (McCabe et al., 1996). Protein concentration was quantitated by the Biorad DC protein detection system, which can detect protein levels in the presence of compounds that inhibit protein measurements in the Bradford method.

3.4 Western blot analysis

Fifty μg of whole cell extracts was loaded per lane on a mini-acrylamide gel. Following electrophoreses, proteins were transferred to PVDF (Biorad) membranes using a semi-dry transfer system (Owl). Protein transfer and size determinations were verified using pre-stained protein markers. Blots were then blocked with 5% nonfat dry milk in TTBS (McCabe et al., 1996) and were subsequently incubated with antibodies specific to different MAP kinase proteins as well as transcription factors (Santa Cruz). Signals were

detected by using an HRP-conjugated secondary antibody and the enhanced chemiluminescence (ECL, Amersham) detection kit.

3.5 EMSA

4μg of nuclear extracts were incubated for 20 minutes at room temperature with ^{32}P-labeled oligonucleotide containing CRE sequence 5' AGA GAT TGC **CTG ACG TCA** GAG AGC TAG 3'. The DNA-protein complexes formed were separated from the free probe on a native 5% gel. DNA binding activity was quantitated by phosphoimager measurement of shifted bands (McCabe et al., 1996).

3.6 Measurement of CRE transactivation by transient transfection

Osteoblasts were transfected with a luciferase reporter plasmid driven by three copies of the CRE-binding sequence fused to TATA-like promoter (PTAL) region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter (CLONTECH Laboratories). MC3T3-E1 cells were plated at a concentration of 100,000 cells per well of a 6 well dish. Twenty four hours after plating, a mix of 1 μg reporter plasmid and 6 μl of lipofectamine (Gibco) were prepared in Optimem (Gibco) and after 45 minutes added to the osteoblasts. After 5 hours, 1 m of 20% FBS α-mem was added to the cells. Fifteen hours later, media is aspirated and a replaced by fresh 10% FBS α-mem. After 24 hours cells were treated with the indicated treatment in the study and harvested and lysed three hours later. Luciferase activity is read immediately using a luminometer. Additional control for these studies include transfection of reporter vector without inserted promoter

elements and co-transfection with a CMV driven luciferase plasmid whose activity is not affected by glucose (activity determined by Promega luciferase assay system).

3.7 Statistical analysis

All statistical analyses were performed using Microsoft excel data analysis program for t test analysis. Experiments were repeated at least three times unless otherwise stated. The autoradiographs shown are of one representative experiment. Values are expressed as a mean ± SEM except where indicated.

4. Results

4.1 Elevation of extracellular sugar specifically enhances p38 phosphorylation

Previously we demonstrated that elevation of extracellular glucose or mannitol alters osteoblast gene expression. Given the important role of MAP kinase in the regulation of transcription factor activities and gene expression, we examined the influence of treating osteoblasts with 16.5 mM extracellular sugar on activation of MAP kinase. Figure 18 and 19 demonstrate that total levels of p38, ERK, and JNK are unaltered after 1 hour of sugar treatment. However, examination of active phosphorylated MAP kinase forms show a 3-4 fold increase in phosphorylated p38 in response to elevated extracellular glucose or mannitol. Activation of the p38 pathway was specific since activation of JNK or ERK was not evident (figure 19). Under these conditions, positive controls (TPA and 600mM glucose) demonstrated that these kinases could be activated in osteoblasts.

4.2 Elevation of extracellular sugar enhances ATF-2 phosphorylation

ATF-2 is a known down stream target for p38 (Zhu and Lobie, 2000). To determine if elevation of extracellular glucose or mannitol enhances ATF-2 phosphorylation, western blot analyses were performed using specific antibodies directed against ATF-2 and phosphorylated ATF-2. Figure 20 demonstrates that while levels of ATF-2 remain constant, phosphorylation of ATF-2, similar to p38, is clearly elevated 3-4 fold one hour after glucose treatment.

4.3 Elevation of extracellular glucose enhances p38 and ATF-2 phosphorylation in time dependent manner

To examine the characteristics of the p38 and ATF-2 phosphorylation in response to osmotic stress, a complete time course was performed with osteoblasts being harvested at 0, 10, 15, 20, 30 and 60 minutes. Figure 21 A shows that the phosphorylation of p38 is evident at 10 minutes, plateaus by 20 minutes and remains activated 60 minutes after glucose treatment. Phosphorylation of ATF-2 is evident within 20 minutes after glucose treatment; this is slower than the phosphorylation of p38 and is consistent with ATF-2 as a downstream target of p38. The response after 24 hours was also examined. Although p38 is considered to be a fast and transient activated kinase, p38 stayed activated even 24 hours after the onset of the osmotic stress (Figure 21 B). As expected, ATF-2 also remained activated at 24 hours.

4.4 Elevation of extracellular glucose enhances p38 and ATF-2 phosphorylation in concentration dependent manner

To determine if the effect on p38 and ATF-2 phosphorylation was concentration dependent, osteoblasts were treated with 2, 4.5, 9.5 and 16.5 mM glucose (final concentration equal to 7.5, 10, 15 and 22 mM glucose respectively) and phosphorylated p38 and ATF-2 levels where examined 1 hour after treatment using western analysis. Remarkably, figure 22 demonstrates that addition of as little as 4.5 mM of glucose is enough to increase p38 phosphorylation in osteoblasts. This level of glucose (10 mM final concentration; 180 mg/dL) is often seen in patients who are diagnosed with diabetes.

4.5 ATF-2 phosphorylation in response to elevation in extracellular sugar is p38 dependent

SB203580 (4-(fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole is a known inhibitor of p38 activity (Sheikh-Hamad et al., 1998). To determine the role of p38 in the phosphorylation of ATF-2 in response to the extracellular sugar elevation we treated osteoblasts with the 10 uM SB203580, 30 minutes prior to glucose treatment and examined responsiveness. Figure 23 demonstrates that SB203580 pretreatment (a concentration suitable for specifically inhibiting p-38 activity (Nadkarni et al., 1999) prior to the addition of 16.5 mM sugar inhibits ATF-2 phosphorylation.

4.6 Increase in CRE DNA binding 1 hour after glucose or mannitol treatment

To demonstrate that the increase in ATF-2 protein phosphorylation is associated with an increase in CRE DNA binding, an electrophoretic mobility shift assay was performed. Figure 24 A shows that indeed there is an increase in CRE DNA binding one hour after glucose or mannitol treatment. To identify the involvement of ATF-2 in the

shifted DNA-protein complex, a supershift study was performed. Nuclear extracts from glucose or mannitol treated osteoblasts were incubated with ATF-2 antibody and ^{32}P labeled CRE DNA consensus probe. Figure 24 B shows that ATF-2 was shifted from the CRE DNA complex. This demonstrates that ATF-2 is a component of the increased CRE DNA binding complex resulting from the elevated extracellular sugar.

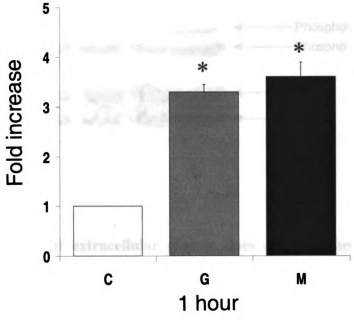
4.7 Elevation of extracellular sugar increases CRE transactivation in osteoblasts, a p38 dependent response

To determine if osmotic stress due to elevation of extracellular sugar can functionally influence CRE transactivation, MC3T3-E1 cells were transfected with a CRE-Luc or CMV-Luc reporter and treated with glucose or mannitol as described in the methods section. Figures 25 demonstrates that increasing extracellular glucose or mannitol enhances CRE transactivation 5 fold compared to the control cells (5.5 mM). CMV-Luc did not show any increase demonstrating the specificity of the CRE induction. To study the role of p38 in the transactivation of CRE, we treated osteoblasts with the 10 uM SB203580 30 minutes prior to glucose treatment and examined responsiveness. Figure 25 demonstrates that p38 inhibition is enough to abolish the CRE transactivation in response to elevated extracellular glucose.

Figure 18. **Elevation of extracellular glucose enhances p38 phosphorylation, a PKC independent response.** A.) Osteoblasts cultured for 11 days were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM. B.) Osteoblasts were pretreated for 30 minutes with 50uM staurosporine (ST) before the sugar treatment. Cells were harvested 1 hour after addition of sugar for whole cell protein extraction. Extracts (50 μg per lane) were separated by 10% SDS-PAGE. Immunoblots were developed using specific antibodies directed against p38 or phosphorylated p38. The blot is a representative of three experiments and contains extracts from control 5.5 mM glucose (C), 22 mM glucose (G) and 5.5 mM glucose + 16.5 mM mannitol (M) treated osteoblasts. Data are the mean ±SEM and are combined from three experiments. *$p<0.03$.

A.


Phospho-p38
p38
C
G
M


Fold increase
5
4
3
2
1
0
*
*
C
G
M
1 hour

B.


Phospho-p38
ST
ST+G
ST+M

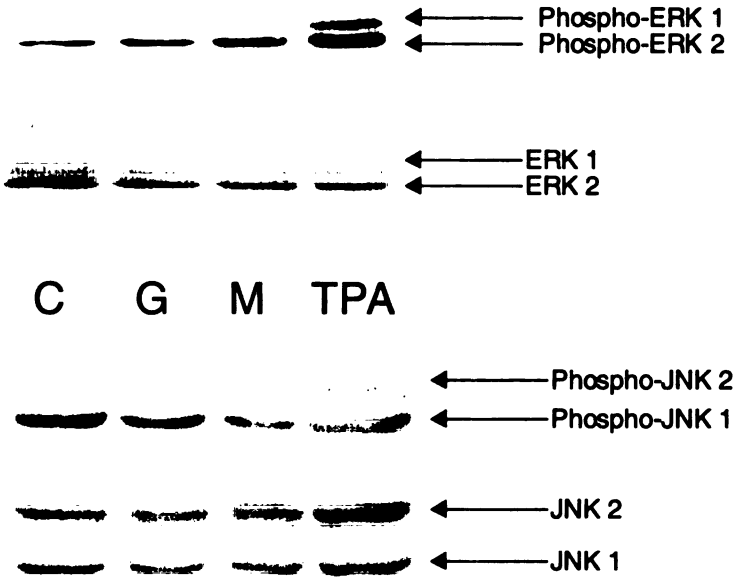
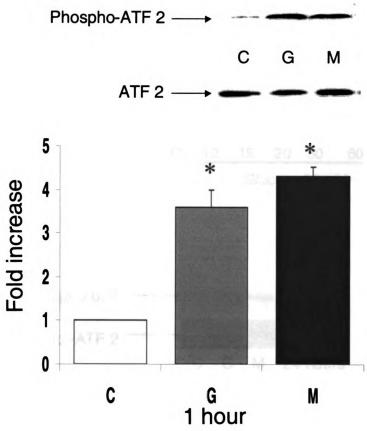


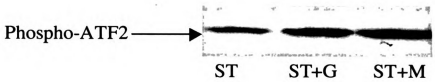
Figure 19. **Elevation of extracellular glucose does not influence ERK and JNK phosphorylation.** Osteoblasts cultured for 11 days were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM. Cells were harvested 1 hour after addition of sugar for whole cell protein extraction. Extracts (50 μg per lane) were separated by 10% SDS-PAGE. Immunoblots were developed using specific antibodies directed against ERK 1-2, JNK 1-2, phosphorylated ERK 1-2 or phosphorylated JNK 1-2. The blot is a representative of three experiments and contains extracts from control 5.5 mM glucose (C), 22 mM glucose (G) and 5.5 mM glucose + 16.5 mM mannitol (M) treated osteoblasts.

Figure 20. **Elevation of extracellular glucose enhances ATF-2 phosphorylation, a PKC independent response.** A.) Osteoblasts cultured for 11 days were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM. B.) Osteoblasts were pretreated for 30 minutes with 50uM staurosporine (ST) before the sugar treatment. Cells were harvested 1 hour after addition of sugar for whole cell protein extraction. Extracts (50 μg per lane) were separated by 10% SDS-PAGE. Immunoblots were developed using specific antibodies directed against ATF-2 or phosphorylated ATF-2. The blots are representative of three separate experiments and contains extracts from control 5.5 mM glucose (C), 22 mM glucose (G) and 5.5 mM glucose + 16.5 mM mannitol (M) treated osteoblasts. Data are the mean ± SEM and are combined from three experiments. *$p<0.05$.

A.


Phospho-ATF 2
C G M
ATF 2
Fold increase
5
4
3
2
1
0
*
*
C
G
M
1 hour

B.


Phospho-ATF2
ST
ST+G
ST+M

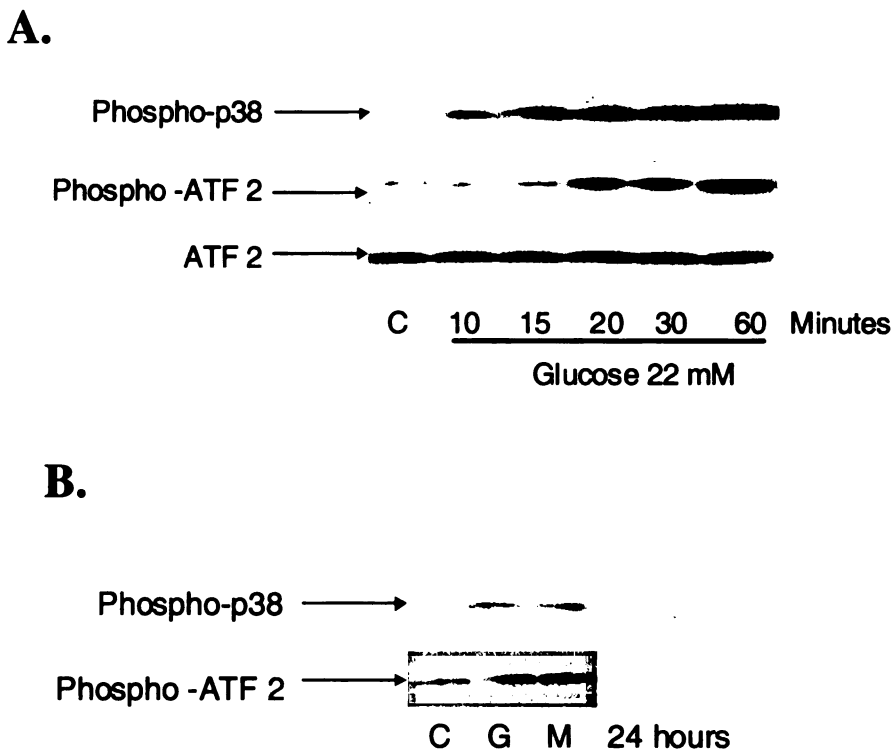


Figure 21. **Elevation of extracellular glucose enhances p38 and ATF-2 phosphorylation in time dependent manner.** Osteoblasts cultured for 11 days were treated with 16.5 mM glucose or mannitol to yield a final concentration of 22 mM. Cells were harvested at the indicated times from 0-60 minutes (A) and 24 hours (B) for whole cell protein extraction. Extracts (50 μg per lane) were separated by 10% SDS-PAGE. Immunoblots were developed using antibodies specific for ATF-2, phosphorylated p38 or ATF-2. The blots are representative of two separate experiments and contain extracts from control 5.5 mM glucose (C), 22 mM glucose (G) and 5.5 mM glucose + 16.5 mM mannitol (M) treated osteoblasts.

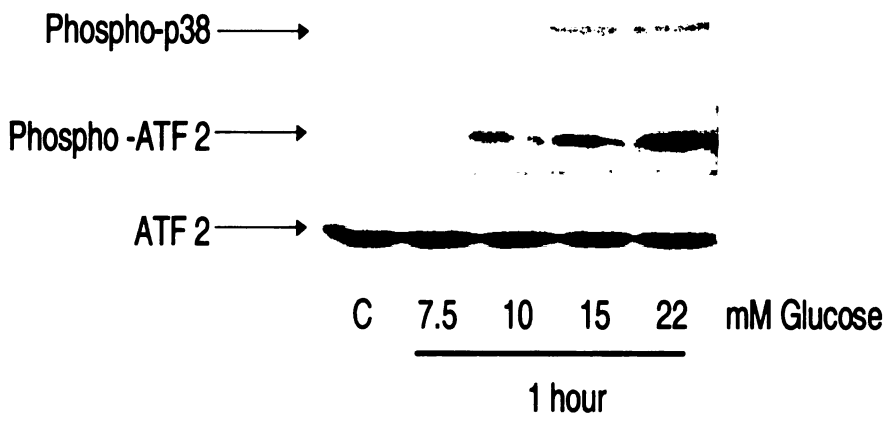


Figure 22. **Elevation of extracellular glucose enhances p38 and ATF-2 phosphorylation in concentration dependent manner.** Osteoblasts cultured for 11 days were treated with 16.5 mM glucose to yield a final concentration of 22 mM. Cells were harvested at the indicated time for whole cell protein extraction. Extracts (50 μg per lane) were separated by 10% SDS-PAGE. Immunoblots were developed using antibodies specific for ATF-2, phosphorylated p38 or ATF-2. The blots are representative of two separate experiments and contains extracts from control 5.5 mM glucose (C), 7.5, 10, 15, and 22 mM glucose treated osteoblasts.

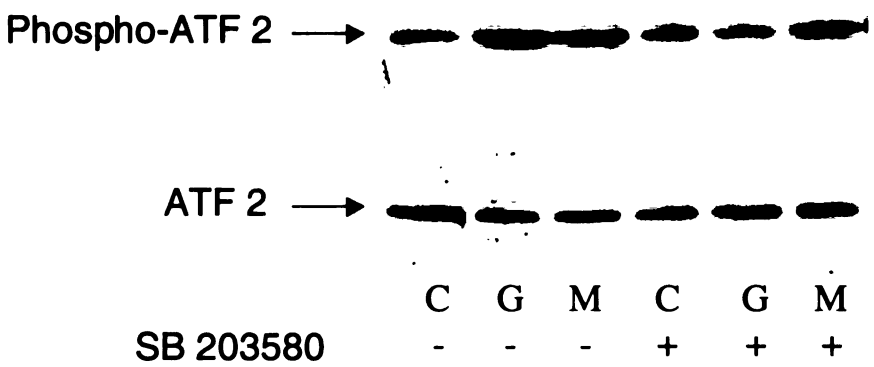
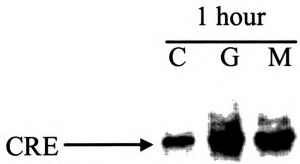


Figure 23. **Extracellular glucose induction of ATF- 2 phosphorylation can be blocked by inhibiting p38 activity.** Osteoblasts were fed every 2 days with media containing 5.5 mM glucose. On day 11 (24 hours after feeding) osteoblasts were pretreated for 30 minutes with 10 μM SB203580. Cells were then treated for 1 hour with 16.5 M glucose (G) or 16.5 mannitol (M) to yield a final concentration of 22 mM. Extracts (50 μg per lane) were separated by 10% SDS-PAGE. Immunoblots were developed using antibodies specific for phosphorylated ATF-2. The blots are representative of two separate experiments and contains extracts from control 5.5 mM glucose (C), 22 mM glucose (G) and 5.5 mM glucose + 16.5 mM mannitol (M) treated osteoblasts.

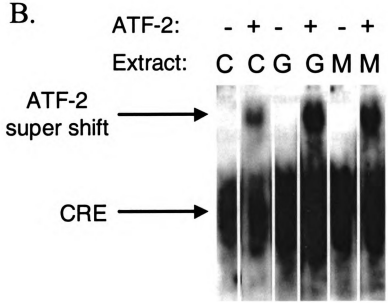
Figure 24. (A.) **CRE binding increases 1 hour after elevation of extracellular glucose or mannitol concentration.** MC3T3-E1 cells were treated with 16.5 mM glucose (G) or mannitol (M) to yield a final concentration of 22 mM. Control cells (C) were untreated. Nuclear extracts were prepared and analyzed as described in methods. 4μg of nuclear extracts were mixed with a ^{32}P-labeled oligonucleotide containing a consensus CRE sequence 5' AGA GAT TGC **CTG ACG TCA** GAG AGC TAG 3' and EMSA was performed. The DNA-protein complexes were separated by 5% polyacrylamide gel. The auto radiographs are representative of 3 separate experiments.

(B.) **Increase ATF-2 binding on CRE DNA element in response elevation in extracellular sugar.** 4μg of nuclear extracts were incubated overnight with a ^{32}P-labeled oligonucleotide containing CRE sequence 5' AGA GAT TGC **CTG ACG TCA** GAG AGC TAG 3' and in the absence (-) or presence (+) of ATF-2 antibody. DNA-protein complexes were separated by 5% polyacrylamide gel electropheresis and visualized by autoradiography.EMSA was performed. n=2.

A.



B.



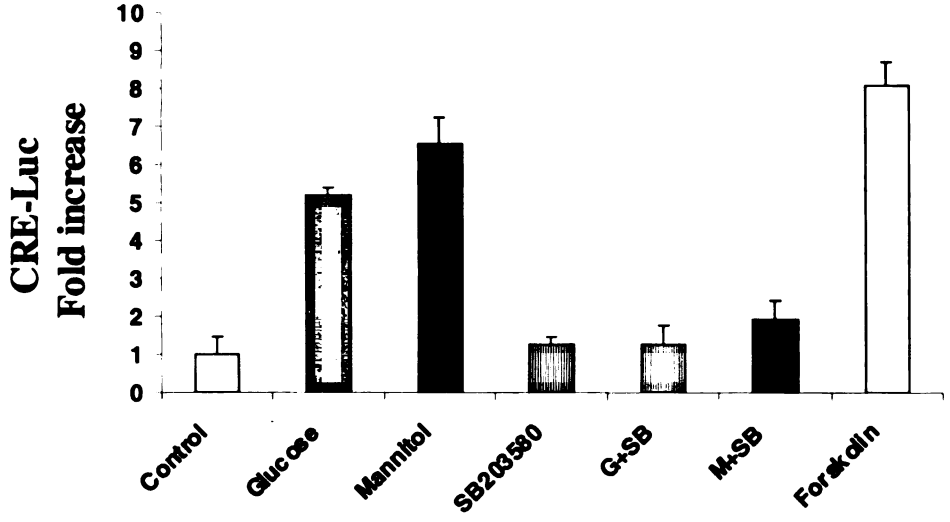


Figure 25. **Elevation of extracellular sugar increases CRE transactivation in osteoblasts, p38 dependent response.** MC3T3-E1 cells were transfected with a CRE-Luc reporter construct. 48 hours after transfection, 16.5 mM glucose or mannitol was added to the plates to yield a final concentration of 22 mM. Reporter activity was measured 3 hours after treatment. SB203580 (10 μm) was used to inhibit p38 activity. Osteoblasts were pretreated for 30 minutes with 10 μm SB203580 before adding the sugar. Data are the mean ± SD of two separate experiments containing triplicate determinations for each condition.

5. Discussion

We have shown previously that osteoblasts respond to an elevation in extracellular glucose through an osmotic response pathway (Zayzafoon et al., 2000). Protein kinases are known to be activated in response to different environmental challenges. We have shown that moderate hyperosmolarity as seen in diabetes affects osteoblasts by increasing *c-jun* expression through a PKC dependent pathway resulting in gene modulation manifested by increase in collagen I and decrease osteocalcin expression. Here, we show that physiologically relevant hypertonicity increases the phosphorylation of p38 MAP kinase after 1 hour of exposure to 22mM glucose or mannitol. This level of hypertonicity failed to activate the other MAP kinases (ERK and JNK) in MC3T3-E1 cells. The specific activation of p38 in response to osmotic stress has been reported before in other tissues (Rizoli et al., 1999; Denkert et al., 1998; Nadkarni et al., 1999). Similar to our results, Duzgun et al., (2000) demonstrate in endothelial cells that p38 phosphorylation is fast and robust in response to a 50 mM increase in glucose or mannitol. Unlike ERK and JNK, p38 induction by osmotic shock occurred as early as 2 minutes and this time point coincides with the activation of the volume regulatory proteins (O'Donnell et al., 1995; O'Donnell, 1993). This observation is consistent with the idea that p38 activation is directly related to the initial cell response to osmotic shock. In this regard, HOG 1, the homologue of mammalian cell p38 in yeast, has been previously demonstrated to be critical element of yeast adaptation to osmotic stress (Takekawa et al., 1997). HOG 1 defective yeast do not proliferate under hyperosmotic condition (Brewster and Gustin, 1994).

To date, the effects of MAP kinases have been mostly attributed to the control of gene transcription via phosphorylation of nuclear transcription. This eventually leads to gene modulation, which plays a part in the cellular response to cell shape change and osmotic stress (Hoffert et al., 2000; Ishida et al., 1999; Schaffler et al., 2000). We have already shown that AP-1 is a crucial component involving increase expression of *c-jun* and modulation of collagen I in osteoblast responsiveness to osmotic stress (Zayzafoon and McCabe, manuscript submitted). Here we show that ATF-2 phosphorylation increases dramatically after one hour treatment with 22 mM glucose or mannitol. ATF-2 phosphorylation is known to increase in response to cellular stresses such as short-wavelength UV (Wilhelm et al., 1995; van Dam et al., 1995) and cellular reperfusion after ischemia (Morooka et al., 1995). ATF-2 target genes include important bone regulatory genes such as tumor necrosis factor α (TNF α) (Tsai et al., 1996), transforming growth factor β (Kim et al., 1992), cyclin A (Shimizu et al., 1998) and *c-jun* (van Dam et al., 1993). These genes are also known to play important roles in the stress response, cell growth and differentiation, and immune response, therefore it is not surprising that osteoblasts respond to osmotic stress by increasing ATF-2 phosphorylation as we show in Figure 20. Although the role of ATF-2 in controlling osteoblast growth and differentiation is still largely unknown, it is known that both parathyroid hormone (PTH) and prostaglandin E_2 (PGE_2) influence the growth and differentiation of osteoblasts mediating CRE (Pearman et al., 1996; Thomas et al., 1996). Phosphorylation of amino acid residues Thr-69 and Thr-71 on ATF-2 has been demonstrated to increase transcriptional activation of ATF-2 (Abdel-Hafiz et al., 1992; van Dam et al., 1995; Tsai et al., 1996). p38 MAP kinase has been shown to

phosphorylate these sites (Raingeaud et al., 1996). Therefore, we expected that the characteristics of ATF-2 phosphorylation to be similar to that of phosphorylated p38. Our results demonstrate that p38 is phosphorylated within 10 minutes after treatment with 22 mM glucose, but there is a lag period of 10 minutes before ATF-2 is phosphorylated. This is consistent with ATF-2 being a downstream target to p38. But what we found to be more interesting was the persistence of phosphorylation of both p38 and ATF-2 after 24 hours. It is known that the ubiquitination of ATF-2 as well as c-Jun is phosphorylation dependent and the early increase in ATF-2 phosphorylation should increase the degradation of this protein and make it unlikely to be phosphorylated by 24 hours (Fuchs and Ronai, 1999). However, it is possible that p38 stress activating protein kinase mediated phosphorylation may stabilize ATF-2, as has been reported in the case with c-Jun (Fuchs et al., 1996; Musti et al., 1997). Phosphorylation of both p38 and ATF-2 when extracellular glucose is between 10 and 15 mM is very interesting because diabetes is diagnosed when glucose levels are between 10 and 15 mM. This demonstrates that osteoblasts are very sensitive to fluctuation in extracellular osmolarity. They elicit an osmotic response as soon as osmolarity has passed a certain threshold which is around 10 mM.

SB203580 is a known specific inhibitor of p38 activity at a low concentration of and 10 μM and is widely used for this purpose (Nadkarni et al., 1999). Using this inhibitor demonstrates that the hypertonicity-induced ATF-2 phosphorylation is p38 dependent. Consistent with our results Nadkarni et al., (1999) showed in hepatic cells (Hep2G) that the hypertonic induction of aldose reductase mRNA as well as osmotic response element (ORE)–driven transactivation are both inhibited by SB203580,

suggesting that p38 MAP kinase mediates the activation of the transcription factors necessary for ORE activation.

Next, we show that hypertonicity increased CRE DNA binding and supershift study shows that ATF-2 is an important part of the complex. In addition, we demonstrate a five fold increase in CRE-Luc activation in response to glucose or mannitol treatment. This activation was shown to be p38 dependent as it was inhibited by the use of SB203580. Similarly, in fibroblasts exposed to stress (heat shock stress in this case) CRE binding and transactivation were increased (Van dam et al., 1992).

Our data indicate that osteoblast are sensitive to small changes in extracellular osmolality and that they activate a p38 MAP kinase pathway. This activation leads to increase in ATF-2 transcription factor phosphorylation. CRE DNA binding and transactivation were also increased in response to hyperosmolarity. These findings suggest that osmotic stress modulates osteoblast signaling pathways contributing to at least one complication of diabetes, osteoporosis.

VI. Summary and Conclusions

Osteopenia and osteoporosis are associated with diabetes. Bone mineral density in patients with both type I and type II diabetes has been reported to be decreased by greater than 10% compared with gender- and age- matched health persons (Levin et al., 1976; Mathiassen et al., 1990; McNair et al., 1988). The objective of this thesis is to understand the mechanisms by which elevation of extracellular glucose can suppress osteoblast function.

We have shown that elevation in extracellular glucose was able to acutely increase the expression of *c-jun* and collagen I while decreasing osteocalcin expression. These results were consistent with a suppression of osteoblast differentiated phenotype. Interestingly, we demonstrate that mannitol can completely mimic the effect of glucose which leads us to hypothesize that the results we are seeing are due to an osmotic response elicited by the osteoblast in response to increased extracellular osmolality. In the process of proving our hypothesis, we have shown that PKC and AP-1 are important for the induction of *c-jun* and col I expression. *In vivo* results support our *in vitro* findings and solidified the hypothesis that osmotic stress is responsible for the decreased mineralization.

Furthermore, we demonstrate that another leucine zipper transcription factor family is involved in the osteoblast osmotic response, the CREB/ATF family. We demonstrate that physiologically relevant hypertonicity increases CRE binding and transactivation. ATF-2, a family member of the CRE/ATF family, is present in CRE binding complex and its phosphorylation is noticeably increased by glucose and mannitol

treatment. p38 MAP kinase was also involved and ATF-2 phosphorylation and CRE transactivation are p38 dependent based on experiments using SB203580 a p38 activity inhibitor.

The combination of our results can be used to hypothesize a novel mechanism employed by osteoblasts in response to physiologically relevant hypertonicity. We have previously shown that hyperosmotic stress induces *c-jun*. The major regulators of c-jun expression are ATF-2 and c-Jun (Van Dam et al., 1995). The promoter for *c-jun* contains two AP-1 sites; a proximal site (TGAGTCA) and a distal site called *jun2*TRE (TTACCTCA) (Angel et al., 1988). *In vitro* binding studies reveal that, in contrast to the consensus AP-1 site which is preferentially targeted by dimers composed of Jun and Fos families, the *jun2*TRE binds heterodimers composed of c-Jun and ATF (-like) proteins. Our data indicate that osmotic stress, indeed, causes an increase in the expression of *c-jun* and it is possible that the two AP-1 sites on the *c-jun* promoter are involved through an initial c-Jun: c-Jun complex binding on the AP-1 consensus followed by c-Jun: ATF-2 heterodimer binding on the *jun2*TRE. The resulting increase in *c-jun* expression could ultimately participate in blocking the function and maturation of osteoblasts by changing their phenotype (increasing COL I and decreasing osteocalcin and perhaps modulating expression of other important genes).

This work provides a novel mechanism in which osteoblast, the bone forming cells, respond to increased extracellular osmolality by activating selected signaling pathways and transcription factors. This leads to the modulation of osteoblast gene expression, which can play a role in altering cell phenotype. This data can be used to increase our understanding of not only diabetic bone disease but also the importance of

osteoblast cell shape in proliferation and differentiation. Understanding the cellular and molecular mechanisms regulating osteoblast phenotype in response to diabetes might aid in the development of drugs, directed at pathways affected by elevated extracellular glucose levels, which can be used to increase bone formation in diabetics and perhaps in the elderly. Such therapies can be used to reduce and possibly prevent the detrimental effects of osteoporosis.

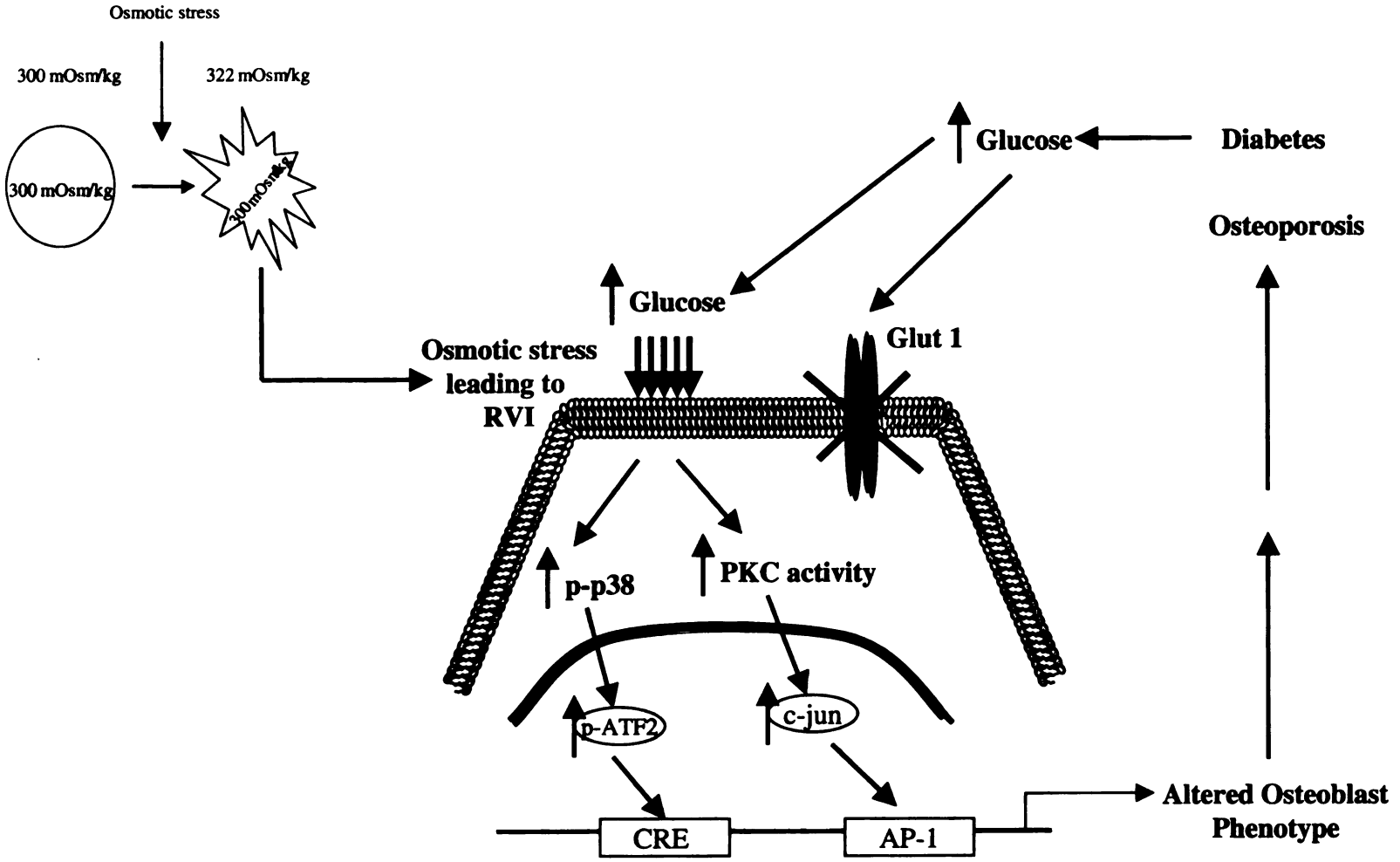


Fig 26. **A model summarizing the work presented in this thesis.**

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