ALTERNATIVE INSULIN MITOGENIC SIGNALING PATHWAYS IN IMMATURE OSTEOBLAST CELL LINES

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Date:

Abstract

Insulin is a mitogen for many cells and commonly signals through the classical, mitogenic Raf-MEK-ERK or metabolic PI3-kinase pathways. Insulin deficiency or type I diabetes causes severe osteopenia. Obese patients with type II diabetes or insulin resistance, a disease associated with defective insulin signaling pathways and high levels of circulating insulin, have increased or normal bone mineral density. The question of whether hyperinsulinemia preserves bone mass is frequently raised. However, there is still a lot of controversy on the role of insulin as an osteoanabolic agent and this question still remains unanswered. A critical role for insulin signaling in bone building osteoblasts has recently been demonstrated with IRS-1 knock-out mice. These mice developed low-turnover osteopenia due to impaired proliferation and differentiation, stressing the importance of osteoblastic IRS-1 for maintaining normal bone formation.

In the present study it was found that insulin does function *in vitro* as an osteoblast mitogen. This was illustrated in three relatively immature osteoblast (MBA-15.4, -15.6 mouse and MG-63 human) cell lines, which responded to insulin with significant increases in proliferation. In the MBA-15.4 preosteoblasts insulin stimulation of proliferation was comparable to the well-described mitogen, TPA. The UMR-106 cell line expresses markers of differentiated osteoblasts, and was much less responsive to insulin treatment. The difference in proliferative potential may be due to differences between spontaneously transformed cell lines, or the stage of cell differentiation.

U0126, a MEK1/2 inhibitor and wortmannin, a PI3-kinase inhibitor, were used to investigate the pathway used by insulin to signal and activate ERK and osteoblast proliferation. In MBA-15.4 mouse preosteoblasts, GF-containing FCS was completely dependent on MEK for DNA synthesis. In contrast, in both MBA-15.4 and more mature MBA-15.6 osteoblasts, insulin-induced proliferation was resistant to the inhibitors alone or in combination. Higher MEK-inhibitor concentrations had no effect, and proliferation was also increased by the inhibitors in several experiments. This indicated that the classical, insulin mitogenic pathway was not involved in MBA-15.4 proliferation. Wortmannin had no effect on either insulin- or 20% FCS-stimulated proliferation, but inhibited activation of Akt/PKB, the metabolic downstream target of PI3-kinase. Insulin signaling to ERK was both MEK- and PI3-kinase- dependent, but this had no effect on proliferation. In contrast, FCS-stimulated ERK activation and proliferation was almost completely dependent on MEK-ERK activation.

Proliferative signaling in the MG-63 human osteoblastic cell line in response to insulin was partially dependent on MEK and partially dependent on PI3-kinase. In contrast, signaling in response to the phorbol ester, TPA, was partially dependent on PI3K but totally dependent on MEK-ERK. This indicates that the signal converges on ERK, suggesting the involvement of a PI3-kinase upstream of a dominant MEK-ERK pathway. The differences found here between mouse and human insulin mitogenic signaling pathways indicate that there may be species differences between osteoblast signaling pathways, with mouse cells being independent and human cells being dependent on MEK for DNA synthesis in response to insulin.

The effects of glucocorticoids on insulin mitogenic signaling in osteoblasts were also investigated, because chronic long-term steroid use results in excessive bone loss. The PTP inhibitor, sodium orthovanadate, reversed GC-impaired TPA- and FCS- induced proliferation in MBA-15.4 and MG-63 preosteoblasts. PTPs, such as SHP-1 and PTP-1B, dephosphorylate and inactivate phosphorylated kinases. Both SHP-1 and PTP1B associated with kinases in the mitogenic signaling cascade of MBA-15.4 preosteoblasts growing rapidly in 10% FCS. Further, SHP-1 co-immunoprecipitated with active, tyrosine phosphorylated ERK, which may indicate that it can dephosphorylate and inactivate ERK. However, since the MEK-ERK or PI3-kinase pathways are not important in insulin-induced proliferation in mouse osteoblasts, the PTPs are unlikely to be role players in the negative regulation of this signaling pathway. This was confirmed by the finding that vanadate was unable to reverse GC-induced decreases in insulin-stimulated DNA synthesis. This suggests that vanadate-sensitive PTPs may not be important in the negative regulation of insulin-induced mouse osteoblast proliferation, and provides further evidence of a novel insulin mitogenic pathway in the MBA-15.4 but not MG-63 osteoblastic cell line.

Abstrak

Insulien is 'n mitogeen vir baie selle en gelei na binding aan die insulien reseptor, intrasellulêre seine via die klassieke, mitogeniese Raf-MEK-ERK of die metaboliese PI3-kinase seintransduksie pad . 'n Insulien gebrek of tipe I diabetes veroorsaak osteopenie. Vetsugtige pasiënte met insulien weestandigheid of tipe II diabetes, 'n siekte wat geassosieer word met foutiewe insulien seintransduksie en hoë vlakke van sirkulerende insulien, het verhoogde of normale been mineraal digtheid (BMD). Die vraag of hiperinsulinemie 'n verlies aan beenmassa teëwerk word dikwels gevra. Teenstrydigheid oor die rol van insulien as 'n osteo-anaboliese stof bestaan egter steeds en hierdie vraag bly dus onbeantwoord. Dat insulien seintransduksie wel 'n kritiese rol speel in beenvormende osteoblaste is onlangs bevestig in studies met muise waarvan die geen vir IRS-1 uitgeslaan is. Hierdie muise ontwikkel 'n lae omset osteopenie weens verswakte proliferasie en differensiasie.

In hierdie studie is gevind dat insulien wel *in vitro* as 'n osteoblast mitogeen kan funksioneer. Dit is in drie relatief onvolwasse (MBA-15.4, -15.6 muis en MG-63 mens) sellyne geillistreer, deur betekenisvolle verhogings in insulien-geaktiveerde proliferasie. In MBA-15.4 pre-osteoblaste is die persentasie verhoging in insulien-gestimuleerde proliferasie vergelykbaar met dié van die bekende mitogeniese forbolester, TPA. Die UMR-106 sellyn het kenmerke van gedifferensieerde osteoblaste, en was baie minder responsief op insulien behandeling. Die verskil in die proliferasie vermoë van die verskillende sellyne kan die gevolg wees van verskille wat bestaan tussen spontaan getransformeerde sellyne of die stadium van sel differensiasie.

'n MEK1/2 inhibitor, U0126 en 'n PI3-kinase inhibitor, wortmannin, is gebruik om die insulien seintransduksie pad noodsaaklik vir die aktivering van ERK en osteoblast proliferasie te bepaal. In MBA-15.4 muis pre-osteoblaste, was fetale kalf serum (FKS)-geinduseerde DNA sintese totaal afhanklik van MEK. Beide die MBA-15.4 en die meer volwasse MBA-15.6 muis osteoblaste was weerstandig teen die inhibitors op hulle eie, of in kombinasie. Verhoogde MEK-inhibitor konsentrasies het geen verdere effek gehad nie en in verskeie eksperimente is 'n verhoging in proliferasie selfs waargeneem met MEK-inhibisie. Hierdie resultate dui aan dat die klassieke insulien mitogeniese pad nie betrokke is in MBA-15.4 gestimuleerde selproliferasie nie. Wortmannin het geen effek gehad op insulien- of 20% FKS-gestimuleerde DNA sintese nie, maar het wel die aktivering van PI3-kinase se metaboliese teiken, Akt/PKB geinhibeer. Insulien seintransduksie aktiveer dus ERK deur beide MEK en PI3-kinase, maar het geen effek op proliferasie gehad nie. FKS-gestimuleerde ERK aktivering en proliferasie was totaal afhanlik van MEK-ERK aktivering.

Insulien-geaktiveerde DNA sintese in die mens MG-63 osteoblaste was gedeeltelik afhanklik van beide MEK en PI3-kinase. Alhoewel TPA ook PI3-kinase kon aktiveer, was dit totaal afhanklik van MEK vir DNA sintese. Dit dui aan dat daar 'n PI3-kinase stroom-op van 'n dominante MEK-ERK seintransduksie pad voorkom. Die verskille wat ons dus waargeneem het in insulien mitogeniese seintransduksie tussen muis en mens, kan aandui dat insulien-gestimuleerde seintranduksie paaie kan verskil van spesie tot spesie. Dit is bevestig met die muisselle wat onafhanklik is en mens selle wat afhanklik is van MEK aktivering vir insulien-geaktiveerde DNA sintese.

Kroniese, langtermyn steroied behandeling kan beenverlies veroorsaak en die effek van glukokortikoide (GK) op die insulien mitogeniese pad in osteoblaste is dus ook ondersoek. Natrium-ortovanadaat, 'n proteien tirosien fosfatase (PTP) inhibitor het GK-verlaagde proliferasie in repons tot beide TPA- en FKS behandeling herstel in MBA-15.4 en MG-63 preosteoblaste. PTPs soos SHP-1 en PTP-1B funksioneer deur gefosforileerde kinases te defosforileer en dus te inaktiveer. Beide SHP-1 and PTP-1B kon assosieer met kinases in die mitogeniese insulien seintransduksie pad van vinnig groeiende MBA-15.4 preosteoblaste in 10% FKS. Verder het SHP-1 ook geko-immunopresipiteer met aktiewe, tirosien-gefosforileerde ERK, wat aandui dat SHP-1 met ERK assosieer om dit te defosforileer en inaktiveer. Die MEK-ERK of PI3-kinase paaie is nie belangrik vir insulien-geaktiveerde seintransduksie in muis osteoblaste nie. Dit is dus onwaarskynlik dat die PTPs 'n rol sal speel in die negatiewe regulering van hierdie seintransduksie paaie. Die ontdekking dat vanadaat nie glukokortikoied-verlaagde insulien-geaktiveerde DNA sintese kan herstel nie, toon dat vanadaat-sensitiewe PTPs nie 'n rol speel in insulien-geaktiveerde proliferasie in muisselle nie. Hierdie bevinding het verder bevestig dat 'n nuwe insulien mitogeniese pad in die MBA-15.4, maar nie die MG-63 selle moontlik bestaan.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
BMD	Bone mineral density
BMP	Bone morphogenetic proteins
BMU	Basic multicellular unit
Cbfa1	Core binding factor α1
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
Dex	Dexamethasone
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
GAP	GTPase activating protein
GC	Glucocorticoids
GEF	Guanine nucleotide exchange factor
GF	Growth factor
GH	Growth hormone
GLUT	Glucose transporter
GR	Glucocorticoid receptor
Grb-2	Growth factor receptor-binding protein-2
GRE	Glucocorticoid-response element
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine triphosphatase
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding proteins
IKK	Ikappa B kinase
IRS	Insulin receptor substrate
JAK	Janus kinase

JNK	c-Jun NH2 terminal kinase
MAPK	Mitogen activated protein kinase
MBD	c-Met binding domain
MEK	MAPK kinase/ ERK kinase
Mr	Molecular weight
nGRE	Negative glucocorticoid-response element
NF-κB	Nuclear factor-kappa B
OPG	Osteoprotegerin
PDK	Phosphatidylinositol-dependent kinase
PH	Pleckstrin homology domain
PI3-kinase	Phosphatidylinositol 3-kinase
PKB	Protein kinase B (also known as RAC or Akt kinase)
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor
PTB	Phosphotyrosine binding domain
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PTP	Protein tyrosine phosphatase
RANK	Receptor activator of NF-κB
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
SH2	Src homology-2
SOCS	Suppressor of cytokine signaling
Sos	Son-of-sevenless
STAT	Signal-transducer and activator of transcription
TGF-β	Transforming growth factor-β

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CHAPTER 1

INTRODUCTION

A. BONE

1. Bone regeneration

The skeleton is a single organ comprised of two tissues, cartilage and bone (For reviews, see Manolagas, 2000; Eriebacher et al, 1995; Watkins et al, 2001). During development and growth the skeleton is continuously regenerated. The bones of a growing child change in shape and size by a process called modeling. During modeling, 100% of the bone surfaces are active, allowing continuous changes in skeletal mass and morphology. Once the skeleton has reached maturity, it undergoes remodeling, a periodic replacement of old bone with new at the same location. Only 20% of the bone surfaces are being remodeled at any given time, resulting in the complete regeneration of the adult skeleton every 10 years (Parfitt, 1994). Bone is usually replaced when it is too old or damaged to carry out its function. In bones that are load bearing, remodeling most likely serves to repair fatigue damage and to prevent excessive aging.

1.1 Remodeling by the basic multicellular unit (BMU)

Two cell types occur in bone, osteoblasts, the bone forming cells and osteoclasts which resorb bone (See Fig.I). In 1965, Hattner et al., observed that new bone is formed in areas which had recently undergone resorption. This remodeling of bone is tightly controlled and is carried out by temporary structures known as basic multicellular units or BMUs (Parfitt, 1994). Each BMU is a discrete packet consisting of osteoclasts in the front and osteoblasts in the rear. Each functions within a defined remodeling cycle separately from other BMUs. The cycle begins when a non-remodeling bone surface becomes activated. The osteoclasts move in, attach to bone in need of replacement, and remove it by acidification and proteolytic digestion. As the BMU advances, osteoclasts leave the resorption site and osteoblasts move in to cover this area with new bone by secreting osteoid which is eventually mineralized into new bone. There are two types of bone, cortical (compact) or cancellous (trabecular) bone. In dense cortical bone the BMU makes a tunnel through the tissue, while in cancellous bone they form a trench by moving across the trabecular surface (Manolagas and Weinstein, 1999). The lifespan of the BMU is 6 months and millions of areas of bone are remodeled at any moment in a healthy human adult. Although the amount of bone resorbed and formed during each cycle of remodeling is tightly balanced, deregulation negatively affects bone mass, causing many metabolic bone diseases.

2. Origin of osteoblasts

Osteoblasts originate from common multipotent mesenchymal stem cells in the bone marrow, which can differentiate into other cell lineages, such as chondrocytes, muscle cells, adipocyes and bone marrow stromal cells (Grigoriadis et al, 1988; Nuttall et al, 1998). Preosteoblasts are found one or two cell layers away from mature osteoblasts (Watkins et al, 2001). Once in the correct position on the bone surface, osteoblasts deposit bone matrix or osteoid. This marks their differentiation and they can then be defined as mature osteoblasts. Osteoid is predominantly composed of type I collagen but also contains other non-collagenous proteins such as osteopontin, osteonectin and osteocalcin. The developing matrix or osteoid is eventually mineralized with calcium and phosphate. Matrix synthesis determines bone volume, while mineralization increases bone density.

During the differentiation process from mesenchymal progenitors, various hormones and cytokines regulate osteoblast differentiation. These regulatory molecules are temporarily expressed and induce nonosteogenic cells to differentiate into mature osteoblasts. Among these, bone morphogenetic proteins (BMPs) not only stimulate normal osteoprogenitors to differentiate into mature osteoblasts, but can also experimentally induce nonosteogenic cells to differentiate into osteoblast lineage cells (Yamaguchi et al, 2000). BMPs stimulate the transcription of the gene encoding core binding factor α1 (Cbfa-1), an osteoblast transcription factor. Cbfa-1 is initially expressed in cells of the mesenchymal condensation (Ducy et al, 1997). Its expression becomes restricted to osteoblasts during development and after birth (Karsenty, 1999). In turn, Cbfa-1 regulates the expression of many osteoblast specific genes and is required to produce a bone extracellular matrix (Wagner and Karsenty, 2001). Besides Cbfa-1, various other transcription factors, such as members of the AP-1 (activator protein-1) complex, are also involved in osteoblast growth and differentiation (Jochum et al, 2001).

3. Origin of osteoclasts

The precursors of the osteoclasts are hematopoietic cells of the monocyte/macrophage lineage. The recent discovery of three proteins has greatly clarified the role that osteoblasts play in osteoclastogenesis (Fig.I; See review by Suda et al, 1999). Firstly, receptor activator of NF-κB (RANK)-ligand, is expressed in committed preosteoblastic cells and T-lymphocytes (Kong et al, 1999). It is initially made as a membrane-bound molecule and subsequently released from the membrane through proteolytic cleavage (Lum et al, 1999). Both soluble and membrane bound RANK-ligand bind with high affinity to RANK (Hsu et al, 1999).

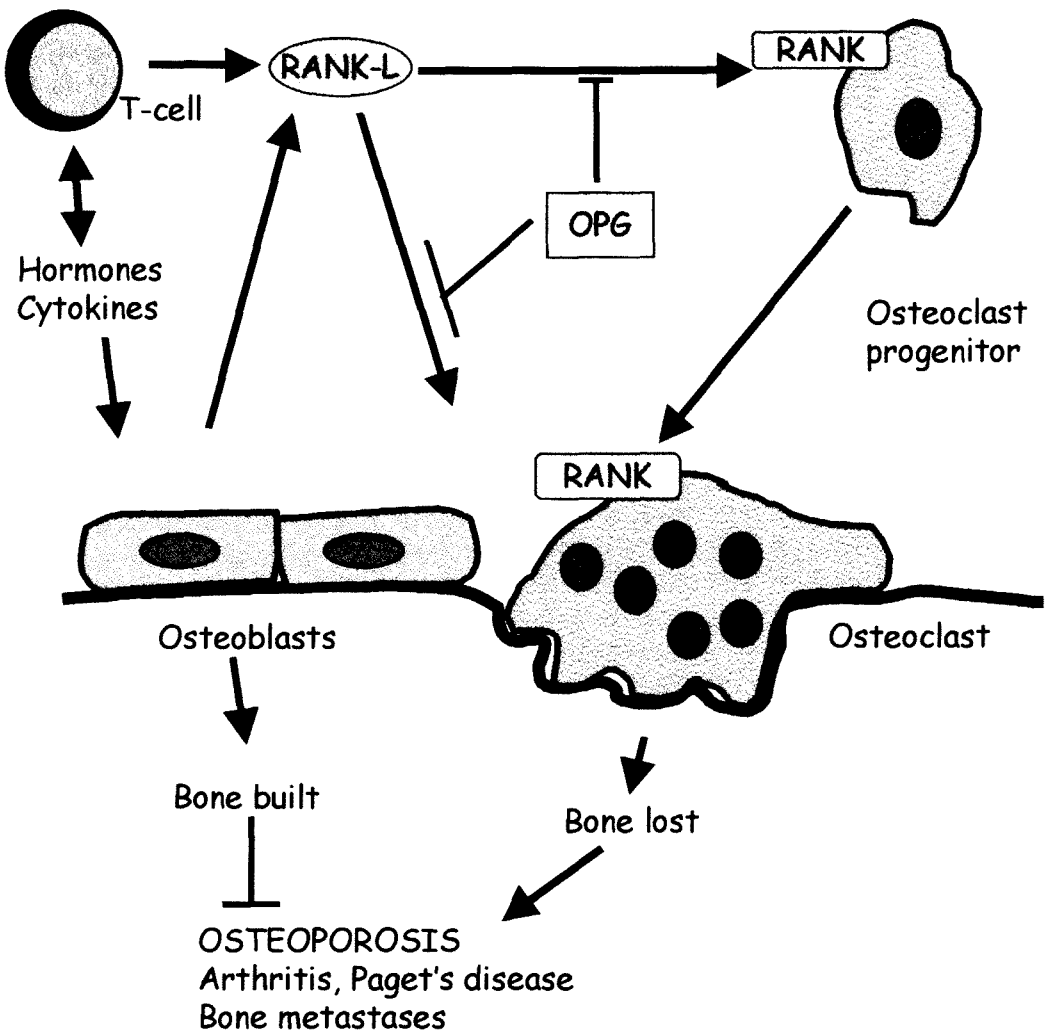

Figure I. Molecular control of bone remodeling and bone disease. RANK-ligand expressed in preosteoblastic cells and T-lymphocytes binds to RANK on osteoclast progenitors. RANK/ RANK-ligand interaction is required for osteoclastogenesis. OPG acts as a decoy receptor and blocks the RANK/ RANK-ligand interaction. (Modified from Kong and Penninger, 2000)

RANK is an integral membrane protein that is expressed in hematopoietic osteoclast progenitors. The RANK/RANK-ligand interaction is essential and together with monocyte macrophage colony stimulating factor (M-CSF), sufficient for osteoclastogenesis (Manolagas, 2000). Osteoclast development is thus dependent on cell-to-cell contact between RANK-ligand expressed on osteoblasts and its receptor, RANK, on osteoclast precursors (Burgess et al, 1999). The third of the three proteins is a soluble receptor called osteoprotegerin (OPG) (Simonet et al, 1997). OPG blocks the RANK/RANK-ligand interaction by acting as a decoy or a neutralising receptor (Yasuda et al, 1998). The ratio of RANK-ligand/ OPG interaction is an important regulatory mechanism in osteoclast maturation and osteoclasts activation *in vitro* and *in vivo* (Lacey et al, 1998). Both the murine and human RANK-ligand genes contain two functional Cbfa-1 sites, and mutation of these sites abolish the transcriptional activity of the RANK-ligand gene promoter (Manolagas, 2000). Since BMP-2 and -4 stimulate Cbfa-1 expression, it has been postulated that a BMP- Cbfa-1- RANK-ligand gene expression cascade exists in cells of the bone marrow/ osteoblastic lineage (Abe et al, 2000). This cascade may control the rate of bone regeneration and maintain the continuous supply of osteoblasts and osteoclasts.

4. Death of osteoblasts and osteoclasts

The average life span of the BMU is 6 months (Manolagas and Weinstein, 1999). Human osteoclasts and osteoblasts have an average lifespan of about 2 weeks and 3 months, respectively. After osteoclasts have eroded to a particular distance, they die and are quickly removed by phagocytes. Most of the osteoblasts (65%) that assembled originally at the remodeling site also die (Jilka et al, 1998). The remaining osteoblasts are converted to lining cells that cover quiescent bone surfaces or they become embedded in the mineralized matrix as osteocytes (Rodan, 1992). Both osteoblasts and osteoclasts die by apoptosis. However, the selective regulation and mechanistic pathways that initiate apoptosis during the bone turnover processes are still not understood in any detail (Hock et al, 2001).

5. Bone disease

To maintain total bone mass, bone formation (osteoblast activity) and resorption (osteoclast activity) must be balanced. An imbalance can result in the formation of either too much bone (osteopetrosis) or too little bone (osteoporosis). Defects in osteoclast differentiation or function are associated with multiple genetically inherited or acquired diseases, all characterized by an arrest of bone resorption (Lazner et al, 1999). Clinical disorders in which bone resorption is increased include Paget's disease, postmenopausal osteoporosis and bone changes secondary to cancer (Kong and Penninger, 2000). Bone loss occurs in all individuals (males and females) after mid-life and is part of the natural aging process (Mundy, 2000). However, osteoporosis is more common in elderly women than in men

(Rizzoli et al, 2001). Possible reasons for the difference is that women have less bone mass at their peak (young adult), which in females accelerates at menopause because of estrogen deficiency (Riggs et al, 1998). Men do not experience a period of accelerated bone loss (menopause) and lose bone more slowly (Bilezikian et al, 1999). Only in acute hypogonadism is bone loss in men rapid, similar to menopausal bone loss in women (Mauras et al, 1999). Postmenopausal osteoporosis also referred to as type I osteoporosis is the most common disorder of bone. Type II or senile osteoporosis occurs mostly in individuals over 70 years of age (Cohen and Roe, 2000). The disease occurs when too little of both bone formation and resorption causes a gradual shrinking of bone mass which is slow and age related (Wick et al, 2000). Reduced bone resorption or osteopetrotic diseases are relative rare in humans e.g. pycnodysostosis due to Cathepsin K deficiency (Saftig et al, 1998). The bone marrow is progressively replaced by the bone extracellular matrix that is continuously deposited by the osteoblasts. Since there is an increase in matrix formation, osteopetrotic bones are characterized by a much denser appearance than normal. Although several animal models are available to study osteopetrosis, there are limitations to using these models when looking for possible therapies (McLean and Olsen, 2001). The human equivalents of the various animal mutations have not been identified (Lazner et al, 1999). In addition to Type I and Type II osteoporosis, prolonged and high dose treatment with glucocorticoids (GCs) is the third leading cause of osteoporosis.

B. GLUCOCORTICOID-INDUCED OSTEOPOROSIS

1. Introduction

The introduction of GC drugs into clinical practice has provided treatment for a diverse group of conditions including asthma, rheumatoid arthritis and several other inflammatory diseases. However, reports of fractures in patients receiving steroid treatment soon appeared, and GC-induced osteoporosis has remained a clinical problem ever since (Reid, 1997). The loss of bone is biphasic, with a rapid initial phase of about 12% during the first 6-12 months, followed by a slower phase of about 2-5% each year. Cortical and cancellous bone are lost, but steroids affect the axial skeleton more severely, manifested by spontaneous fractures of the vertebrae or ribs (Manolagas, 2000). Osteonecrosis, can also occur, which causes collapse of the femoral head in about 25% of patients (Pritchett, 2001).

2. Molecular mechanisms of glucocorticoid action

Effects of lipophilic GCs (cortisol in man, guinea pig and rabbit; corticosterone in rodents) are mediated by an intracellular receptor, the glucocorticoid receptor (GR) (Hollenberg et al, 1985). GR belongs to the nuclear hormone receptor superfamily (Beato et al, 1995). After passing the plasma membrane, GCs bind to GR. Steroid hormone receptors together with heat shock proteins are trapped in an inactive cytosolic complex, in the absence of a ligand. Upon ligand binding, associated heat shock proteins are released and the ligand-bound receptor translocates into the nucleus (Reichardt and Schütz, 1998). Once in the nucleus, the GC-GR complex can utilize several different mechanisms in order to influence gene expression (Reviewed by Aranda and Pascual, 2001). Upon binding of the activated GR as a homodimer to the glucocorticoid-response elements (GREs), or negative GREs (nGREs) it can induce or repress the transcription of target genes (Sakai et al, 1988). Some genes lacking GREs or nGREs in their promoters can still be affected by GCs. By "transcriptional cross-talk" of GR with other transcription factors, the gene expression pattern within cells can be influenced by GR without its binding to DNA (Göttlicher et al, 1998). Well known examples include the GR and AP-1 transcription factor interacting on target gene promoters which contain binding sites for only one class of factors, either AP-1 or steroid hormone receptors (Wei and Vedeckis, 1997). Negative interference of both factors with each other's activity has been observed. For example when AP-1 is bound to its cognate DNA-recognition site, the GR can modulate AP-1 activity through protein-protein interaction. Depending on the composition of the AP-1 complexes they can either co-operate or antagonize transcription by the GR. The GR represses AP-1 activity when it is composed of c-Fos and c-Jun (Yang-Yen et al, 1990). However, synergism is possible under cell–specific conditions and when AP-1 is a homodimer of c-Jun (Diamond et al, 1990).

3. Actions of glucocorticoids on bone

GCs have profound effects on bone metabolism, acting at many sites (Fig.II). GCs increase bone resorption and decrease bone formation, leading to a decrease in bone mass and a higher risk of fractures (Canalis, 1996; Ziegler and Kasperk, 1998). *In vivo*, a cause of excessive bone resorption is enhanced stimulation of parathyroid hormone (PTH) secretion or activity (McSheehy and Chambers, 1986). Although GCs can directly stimulate PTH secretion, PTH secretion is mainly stimulated by the negative serum calcium levels caused by a decrease in intestinal calcium absorption and increasing urinary calcium secretion (Au, 1976; Suzuki et al, 1983). GCs increase osteoblast PTH receptor expression, enhancing their responsiveness to PTH. Osteoblast recruitment and differentiation is inhibited by high-dose GCs. This is associated with reduced collagen type I production and osteocalcin synthesis, which are markers of osteoblastic function (Wong et al, 1990; Delany et al, 1995). Collagenase 3 production may be increased, which degrades collagen type I, a structural protein of the bone matrix (Delany et al, 1995).

GCs can indirectly affect bone growth by opposing the effects of osteoblast growth factors such as insulin-like growth factor (IGF)-1, IGF-2 and transforming growth factor-β (TGF-β). IGF-1, -2 and -3 are secreted by mature osteoblasts and stimulate osteoblast proliferation and differentation through specific membrane receptors (Thomas et al, 1999). The activity of IGF is regulated by six IGF-binding proteins (IGFBP), which are all expressed by osteoblasts (Rechler and Clemmons, 1998). IGFBP-5 enhances the effects of IGF-1 and IGFBP-6 inhibits IGF-2. GCs decrease IGFBP-5 synthesis and increases IGFBP-6 production, thus decreasing the amounts of IGF-1 and IGF-2 available to bone cells (Canalis, 1998). GCs inhibit TGF-β activity and suppress binding to its receptor TGF-β type I on osteoblasts. In addition, binding sequences for the osteoblast transcription factor Cbfa-1, occur in the TGF-β type I receptor promoter (Ji et al, 1998). This may explain the observation that GCs suppress Cbfa-1 causing a decrease in TGF-β type I receptor expression and activity (Chang et al, 1998). The decrease in osteoblast numbers and the increase in osteoclast activity produce a marked decrease in bone formation. However the diverse and complex effects of GCs on bone metabolism are still incompletely understood, mainly due to differences in experimental outcomes in different systems and culture conditions (Ishida and Heersche, 1998).

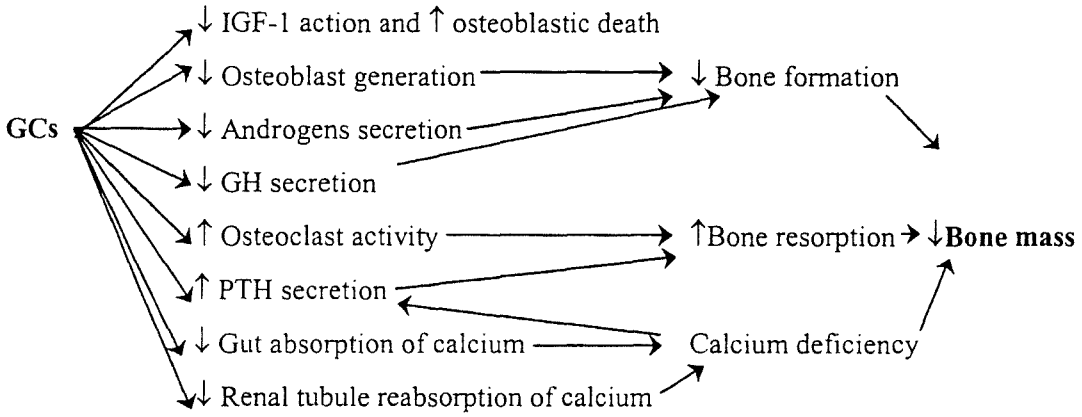

Figure II. Sites and mechanism of GC effects on bone metabolism in humans. (Modified from Manelli and Giustina, 2000).

4. Glucocorticoids and bone formation

Impairment of osteoblast numbers is one of the main contributors to GC-induced bone loss. The demonstration of GR in osteoblasts, suggested a direct effect of GCs on osteoblast function (Feldman et al, 1975; Chen et al, 1977). *In vitro,* the effects of GCs are dose-dependent. Low concentrations stimulate osteoblast differentiation and increase matrix synthesis in mature osteoblasts (Shalhoub et al, 1992). However, high-dose GCs cause inhibition of type I collagen and alkaline phosphatase gene expression and decreased action of growth factors (GF), in particular IGF-I (Canalis, 1996). *In vivo,* prolonged treatment with even physiological GC doses causes osteoporosis (Ebeling et al, 1998). The constant long-term GC administration results in supraphysiological GC levels and a loss of the normal circadian pattern of cortisol secretion. This is comparable to the inhibitory effects seen *in vitro* when GC dose is increased beyond optimal concentrations (Ishida and Heersche, 1998).

Hulley et al., (1998) have shown that GC treatment of a preosteoblastic cell line induces decreased response to GFs of the mitogenic Raf-MEK-ERK kinase cascade, impairing osteoblast proliferation (Table 1). Further, since the osteoblast transcription factor Cbfa-1 is downstream of extracellular signal regulated kinase (ERK), decreased ERK activity would negatively affect Cbfa-1 transcription and bone growth (Xiao et al, 2000). The protein tyrosine phosphatase (PTP) inhibitor, sodium orthovanadate, reversed the effects of Dexamethasone (Dex), suggesting the involvement of PTPs, possibly by GC-induced up-regulation (Hulley et al, 1998). Hulley et al., (2001) confirmed this result in a GC-induced osteoporotic rat model. Daily vanadium supplementation increased osteoblastic bone formation, thus improving bone mineral density (BMD) and bone strength (Hulley et al, 2001). These

studies indicated that high dose GCs inhibit the mitogenic cascade and that the PTP inhibitor vanadate is effective in preventing this. Vanadate would act effectively either if GCs up-regulate PTPs causing excessive or rapid dephosphorylation of kinases, or if specific tyrosine phosphorylated substrates are down-regulated by GC, resulting in a net imbalance of kinase vs PTP activity. Clinically, vanadate has been shown to improve insulin sensitivity and decrease glucose levels in both diabetic animals (Meyerovitch et al, 1987; Brichard et al, 1988) and humans (Cohen et al, 1995; Goldfine et al, 1995). Possible targets of vanadate action are the PTPs, PTP-1B and SHP-1. PTP-1B is a negative regulator of insulin signaling (Goldstein et al, 2000), while SHP-1 has been reported to dephosphorylate the insulin receptor (Bousquet et al, 1998) and to be up-regulated by GCs (Cambillau et al, 1995). By preventing dephosphorylation of specific tyrosine phosphorylation sites (i.e. insulin receptor and insulin receptor substrate-1), vanadate effectively turns off a negative switch, providing a prolonged active state of important insulin signaling events.

Recent findings indicate that GCs also promote apoptosis of the bone forming cells. Mice treated with GCs show a three-fold increase in osteoblast apoptosis, while 28% of the osteocytes died by apoptosis (Weinstein et al, 1998). Preliminary studies indicate that overexpression of the Bcl-2 gene reduced GC-induced apoptosis (Bellido et al, 1998). A possible mechanism is that GCs, by lowering the levels of anti-apoptotic protein Bcl-2 and raising levels of Bax, an apoptosis-inducer, apoptosis in osteoblasts is promoted (Mocetti et al, 2001). Since GFs such as insulin have pronounced anti-apoptotic effects, decreased response or "GF resistance" caused by GCs would also favour apoptosis. An additional loss of osteoblasts may be explained by increased fat formation in the bone marrow of mice and humans with GC excess as a result of increased expression of peroxisome proliferator-activated receptor (PPAR)-γ2, a transcription factor that induces terminal adipocyte differentiation while suppressing osteoblast differentiation (Lecka-Czernik et al, 1999).

Table1. Cellular changes and possible agents in glucocorticoid-induced osteoporosis

Cellular changes	Possible agents
↓ osteoblastogenesis	decreased Cbfa-1 and TGF-β type I receptor; decreased BMP2 and IGF-I action, #GF resistance
↑↓osteoclastogenesis*	increased PPARγ2 expression
↑ bone marrow adipocity	
↓lifespan of osteoblasts	decreased Bcl-2/ BAX ratio, #GF resistance
↓ lifespan of osteocytes	decreased Bcl-2/ BAX ratio, #GF resistance

*Osteoclast numbers may increase transiently at the early stages of steroid therapy, but decrease subsequently; osteoclast activity may increase due to increased PTH (Table modified from Manolagas SC and Weinstein RS, 1999; #Hulley et al, 1998)

5. Treatment (Possible bone builders)

Many more drug therapies are available to reduce bone resorption, than to stimulate bone formation. For example, hormone replacement therapy, bisphosphonates and fluoride are commonly prescribed for treatment of Type I osteoporosis (López, 2000). For most anabolic agents the side effects outweigh the benefits of modest increases in bone mineral density. For example, fluoride treatment produces increases in trabecular bone volume and lumbar spine BMD (Lems et al, 1997; Farley et al, 1983). However, at high doses it interferes with normal mineralization, increasing bone fragility (Hedlund and Gallagher, 1989). Anabolic steroids and testosterone treatment have also been shown to increase bone mass (Libanati and Baylink, 1991). However, side effects include prostate cancer and severe cardiac and hepatic diseases (Avila et al, 2001; Farrell et al, 1975).

It is clear that the ideal therapy for the treatment of GC-induced bone loss would be the use of agents that enhance bone formation. Other possible bone anabolic agents include growth hormone (GH) and PTH. GH secretion declines with age both in humans and in rats. In old female rats GH injections and mild exercise in combination modulate and increase further the formation and strength of cortical bone (Oxlund et al, 1998). In a young (2 months old) rat model, GH increased growth in GC-injected animals, but the effect was however, dose-dependently decreased by GC-administration (Ørtoft et al, 1999). When female rats (3½ months old) were treated concomitantly with GCs and GH, the GH failed to counteract the decreased bone formation and increased bone resorption induced by protracted treatment with a high dose of GCs (Ørtoft and Oxlund, 1996).

Although continuous administration of PTH results in bone resorption, it has been established that pulsatile administration by daily injection of low dose PTH and the PTH-related protein (PTHrP) increases osteoblast number, bone formation rate and bone mass (Schmitt et al, 2000). Recently it was reported that daily injections of PTH in mice increase the life-span of mature osteoblasts by preventing their apoptosis (Jilka et al, 1999). This study indicated that PTH exerts its protective effect not by stimulating osteoblast proliferation but by preventing osteoblast apoptosis. Bisphosphonates and calcitonin also have been developed as potent inhibitors of bone resorption. As with PTH, data indicates that inhibition of osteoblast apoptosis is the most likely mechanism of the anabolic effect of these agents (Plotkin et al, 1999). Thus, *in vitro* bisphosphonates and calcitonin exert antiapoptotic effects on osteocytic cells and mature osteoblasts, and *in vivo* bisphosphonates prevent osteocyte and osteoblast apoptosis induced by GC excess.

By increasing osteoblast lifespan, more bone formation can take place. However, none of these agents directly stimulate osteoblast proliferation, and there is still a need for development of drugs which increase osteoblast numbers by stimulating the precursor population.

5.1 Vanadate

A study in 1985 involving vanadium supplementation in the drinking water of an experimental diabetic rat model led to the discovery of vanadium's insulin mimetic and anti-diabetic potential (Heyliger et al, 1985). Sixteen years later, vanadium compounds have been tested extensively as treatment for diabetes in both animals and humans. It was found *in vivo* that vanadium requires the presence of insulin to elicit its effects on metabolism, suggesting that vanadium acts as an insulin enhancer (Poucheret et al, 1998). The general opinion is that vanadium acts through inhibition of PTPs, that dephosphorylate the insulin receptor or post-receptor level in insulin signaling pathways (discussed in section C). The importance of the involvement of a specific phosphatase was confirmed with the PTP-1B knockout mice (Elchebly et al, 1999). These mice showed increase insulin sensitivity and lower fed blood glucose and insulin levels. The PTP-1B (-/-) were resistant to weight gain when fed a high fat diet compared to the PTP-1B (+/+) littermates that rapidly gained weight. Theoretically, as inhibitors of PTPs, vanadium should enhance all phosphotyrosine-dependent signals, such as those involved in GF response (Hulley et al, 1998). Vanadate supplementation in GC-treated rats does prevent bone loss, increasing osteoblast numbers and bone formation parameters (Hulley et al, 2001). However, the precise molecular targets of vanadate in bone are not yet known.

C. INSULIN SIGNALING

1. Introduction

Insulin is clearly important for bone health. The IRS-/- mouse develops severe osteopenia (Ogata et al, 2000), as do type I diabetic humans (Hough, 1987) and rats (Hough et al, 1981). The insulin mimetic, vanadium, also builds bone in the low formation state induced by glucocorticoids (GC) (Hulley et al, 2001). However, insulin mitogenic signaling in osteoblasts is not well described.

2. Insulin receptor

Insulin is secreted by the β-cells of the pancreas and its primary function is to regulate glucose homeostasis. β-cells are sensitive to increasing blood glucose concentrations. Biological actions of insulin are initiated when insulin binds to its cell surface receptor (Fig.III). Variable numbers of the receptor are expressed in almost all mammalian tissues, with the highest concentration (>300, 000 receptors per cell) being found on two of insulin's major target tissues, adipocytes and liver (Cheatham and Kahn, 1995). However, insulin can also bind to and activate other receptors such as the IGF-1 receptor. IGF-1 is produced in multiple tissues, but is secreted predominantly in the liver and acts to stimulate tissue growth and differentiation. The insulin and IGF-1 receptor are similar in overall structure, but their binding affinity for their heterologous receptors are approximately 100-fold less than for their own receptors (Nystrom and Quon, 1999). It was always believed that the mitogenic effects of insulin were only mediated through the IGF-1 receptors. However, it was proved that in some cells insulin mitogenic signaling may be mediated by both its own and IGF-1 receptors (Ish-Shalom et al, 1997).

2.1 Structure

The insulin receptor and highly homologous IGF-1 receptor are members of a family of ligand-activated receptor tyrosine kinases (RTK) (LeRoith et al, 1995). They are subdivided into members of class II receptors because of the cysteine-rich motifs in their extracellular α–subunits and are disulfide-linked heterotetramers (Ullrich and Schlessinger, 1990). The $\alpha_2\beta_2$-heterotetramer structure consists of two extracellular α-subunits and two transmembrane β-subunits held together by disulfide bonds (Navarro et al, 1998). Although structurally and functionally related, the insulin and IGF-1 receptor modulate different responses within the cell. IGF-1 has been implicated mostly in mitogenic functions and insulin in metabolic actions.

2.1.1 The α-subunit

The α-subunit has a molecular weight (Mr) of approximately 135, 000 Daltons and is located entirely extracellularly (Kasuga et al, 1982). It has an N-terminal domain responsible for high-affinity insulin binding (Gustafson and Rutter, 1990). The subunit has a total of 37 cysteine residues encoded by exons 3-5. Cysteine 524 is involve in α-α disulfide bonds, covalently linking two α-β heterodimers (Sparrow et al, 1997). Two insulin receptor isoforms exist, which arise by alternative splicing of the mRNA. The isoforms differ in the absence or presence of exon 11, a 12-residue segment in the most carboxy-terminal domain of the α-subunit (Sesti et al, 1995). In the absence of insulin, the α-subunit maintains an inhibitory influence over the tyrosine kinase in the β-subunit, inhibiting an otherwise constitutively active kinase (Shoelson et al, 1988).

2.1.2. The β-subunit

The β-subunit has an apparent Mr of 95,000 Daltons and anchors the receptor in the membrane (Lönnroth, 1991). It consists of a short extracellular domain, a transmembrane domain, and an intracellular domain that contains a tyrosine-specific protein kinase. Tyrosine kinase activity is required for insulin action (Kasuga et al, 1982). Three tyrosine residues, 1158, 1162 and 1163, are major sites of autophosphorylation and comprise the kinase regulatory domain (Wilden et al, 1992). Both the carboxy-terminus and juxtamembrane region also contain tyrosine phosphorylation sites, but these do not appear to be important for activation of the kinase (Cheatham and Kahn, 1995).

2.2. Insulin receptor substrate (IRS) family members

Upon ligand binding, most tyrosine kinase receptors undergo autophosphorylation, creating high-affinity binding sites for various molecules that contain src homology-2 (SH2) domains. However, apart from a possible role for Shc, these interactions are not critical for insulin signaling (Yamauchi and Pessin, 1994). There are a number of substrates for the insulin receptor tyrosine kinase such as insulin receptor substrate-1 (IRS-1), IRS-2, -3, -4, and Gab-1 that provide an interface between the insulin receptor and downstream SH2-domain containing signaling molecules (Nystrom and Quon, 1999; Lehr et al, 2000). The IRS proteins have several features in common, including a well-conserved pleckstrin homology (PH) domain, followed by a phosphotyrosine binding (PTB) domain, that are important for efficient coupling to the activated insulin receptor (Whitehead et al, 2000). The PTB domain is completely absent from Gab-1 but it contains a c-Met binding domain (MBD) providing a mechanism for receptor-substrate coupling (White, 1998). The carboxy-terminal of IRS contains multiple tyrosine-rich motifs that undergo phosphorylation by the insulin receptor and serve

as docking sites for SH2-domain containing proteins. While there is considerable evidence for a direct role of IRS-1 and IRS-2 in insulin's metabolic actions, the role of IRS-3 and IRS-4 are less clear. Results from knock-out mice indicated that IRS-1 (-/-) mice have retarded growth and are insulin resistant (Araki et al, 1994). Osteoblastic IRS-1 is essential for maintaining bone turnover. Mice lacking the IRS-1 gene showed reduced proliferation and differentiation of osteoblasts and impaired osteoclastogenesis, resulting in low-turnover osteopenia (Ogata et al, 1994). IRS-2 (-/-) mice develop diabetes due to a combination of insulin resistance and failure to develop compensatory response to β-cells (Withers et al, 1998). There are also tissue- specific differences in the roles of the IRS proteins in mediating insulin action, with IRS-1 being more prominent in skeletal muscle and IRS-2 in liver (Kido et al, 2000). It appears that IRS-3 and IRS-4 have less prominent roles in glucose metabolism than IRS-1 and -2, raising the question as to whether these two molecules play any role in insulin and IGF signaling (Fantin et al, 2000; Liu et al, 1999).

3. Insulin metabolic signaling

Once insulin binds to and activates its receptor tyrosine kinase, insulin signaling pathways diverge. One pathway proceeds through the insulin receptor substrates IRS-1 and IRS-2 and depends on the activation of the enzyme phosphatidylinositol 3-kinase (PI3-kinase). Another pathway goes through Grb2/Sos, leading to activation of the MAP kinase (ERK) cascade. Insulin produces most of its metabolic actions through the PI3-kinase pathway (See Fig.III).

3.1 Mediators of insulin-regulated glucose transport

A primary function of insulin is to control blood glucose concentrations by stimulating glucose transport into target tissues such as muscle and adipocytes. Two types of glucose transporters are responsible for glucose uptake into the body. The sodium-linked glucose transporters are mostly localized in the intestine and kidney and are not known to be regulated by insulin (Shepherd and Kahn, 1999). Glucose uptake into all other types of tissue is accomplished by the facilitative glucose transporters referred to as GLUT1-5 (Cheatham and Kahn, 1995). GLUT4 is the only major insulin-regulated glucose transporter and is located primarily in adipose tissue and muscle cells. In the absence of insulin, almost all of the GLUT4 is found in an intracellular storage pool (Holman and Sandoval, 2001). In response to insulin a rapid increase in the rate of glucose uptake is induced. This occurs primarily as a result of the translocation of vesicles containing GLUT4 to the plasma membrane. The vesicles fuse with the plasma membrane, causing a 10-40 fold increase in GLUT4 concentration and the rate of glucose transport into the cell also increases (Cheatham, 2000).

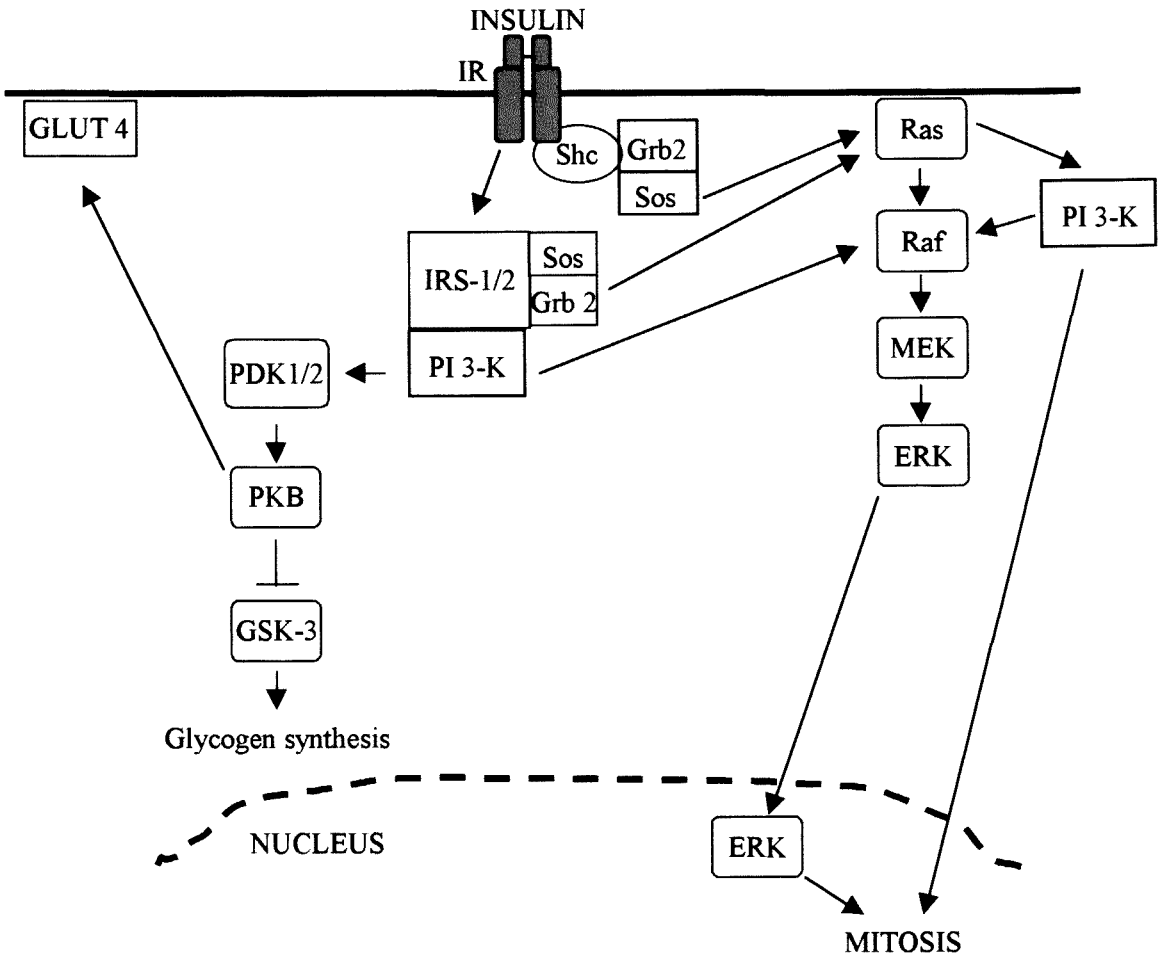

Figure III: Insulin signaling pathways. (A) Metabolic pathway: Insulin binding to its receptor results in receptor autophosphorylation and tyrosine phosphorylation of insulin receptor substrates (IRS). IRS associates with the regulatory subunit of PI3-K. PI3-K lipid products activate PDK1/2, which activates Akt/PKB. PKB deactivates GSK-3, leading to activation of glycogen synthesis. Activation of PKB also results in translocation of GLUT4 vesicles to the plasma membrane.
(B) Mitogenic pathway: Other signaling molecules can associate with IRS, such as Grb2. Grb2 is constitutively associated with Sos, which activates Ras, leading to activation of Raf, MEK, ERK and translocation of ERK to the nucleus. PI3-K can lie downstream of Ras or signal to mitosis through Raf (Modified from Bevan, 2001).

3.2 PI3-kinase and the metabolic effects of insulin

The first downstream molecule that was shown to associate with IRS-1 is PI3-kinase (Sun et al, 1991; Folli et al, 1992). This enzyme is composed of a 110kDa catalytic (p110) and a 85kDa regulatory (p85) subunit. The p85 subunit contains two SH2 domains, through which it associates with tyrosine phosphorylated IRS (Myers et al, 1992). This activates the catalytic function of the associated p110 subunit. This subunit has a lipid kinase activity which phosphorylates the D-3 position of the inositol ring of phosphoinositides, producing phosphatidylinositol 4, 5- bisphosphate [PI-(4, 5) P_2] and PI-(3, 4, 5) P_3 (Ogawa et al, 1998; Srivastava, 1998).

3.2.1 Glycogen synthesis

Protein kinase B/ PKB (also known as RAC or Akt kinase) is regulated through both localization (Andjelkovic et al, 1997) and phosphorylation (Toker and Newton, 2000). The PI3-kinase lipid products bind to the pleckstrin homology domain of PKB and function to attach cytosolic, inactive PKB to the plasma membrane (Stokoe et al, 1997). PKB undergoes a conformational change allowing phosphatidylinositol-dependent kinases 1 and 2 (PDK1/2), to phosphorylate and activate PKB. This activation requires phosphorylation of serine and threonine residues. PDK1 was shown to phosphorylate threonine 308, while the kinase that controls serine 473 phosphorylation is still unknown, but has been designated PDK2 (Alessi et al, 1997). Phosphorylation of both residues is required for full PKB enzyme activity (Hajduch et al, 2001). Active PKB in turn phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3), leading to activation of glycogen synthase and thus glycogen synthesis (Ueki et al, 1998; Cross et al, 1995).

Interesting findings or exceptions to the classical pathway include the activation of PKB independent of PI3-kinase. Kroner et al., (2000) revealed in platelets a PI3-kinase-independent PKB activation in which protein kinase C (PKC)-α/β regulates the phosphorylation of serine 473 in PKBα. Threonine 450 is constitutively active and does not contribute to PKBα activation (Kroner et al, 2000). Also, in addition to its well established role at the plasma membrane, an active PI3-kinase has been shown in nuclei of different cell types (Martelli et al, 2000). In another study intranuclear translocation of PKB was shown (Borgatti et al, 2000). This indicates that both PI3-kinase and PKB not only act at the plasma membrane but can also themselve move to the nucleus and affect proliferation.

3.2.2 Glucose transport

Another important metabolic response to insulin-stimulated activation of PI3-kinase is GLUT4 translocation and uptake of glucose into the cell. Besides PKB activation, the lipid products of PI3-kinase have been shown to regulate several other intracellular serine/ threonine kinases, including mTOR, p70 S6 kinase and the "atypical" protein kinase C (PKC) isoforms, PKC-λ and PKC-ζ (Cheatham, 2000). Of these, PKB, PKC-λ and PKC-ζ elicit signals that can modulate GLUT4 translocation (Standaert et al, 1999; Tanti et al, 1997).

Data implicating PKB in GLUT4 translocation to the plasma membrane comes from studies involving over-expression of constitutively active/inactive mutants of PKB in cultured cell models. In 3T3-L1 adipocytes, overexpression of constitutively active PKB leads to increase recruitment of GLUT4 to the cell surface (Kohn et al, 1996). Furthermore, a dominant-negative (kinase-inactive) mutant significantly inhibited insulin-stimulated translocation of GLUT4 (Cong et al, 1997). From the literature it seems that PI3-kinase translocates from the cytosol to GLUT4-containing vesicles in response to insulin (Calera, 1998). The PI3-kinase lipid products recruit PKB, promoting translocation of the vesicles to the plasma membrane and stimulation of glucose transport.

Similarly, the involvement of PKC-λ and PKC-ζ in insulin-stimulated GLUT4 translocation has been reported in several cell culture systems. In L6 myotubes, expression of wild-type or a constitutively active PKC-λ stimulated GLUT4 translocation (Bandyopadhyay et al, 2000). In 3T3-L1 adipocytes, expression of kinase-inactive mutants of atypical PKCs inhibited insulin-stimulated GLUT4 translocation, but without any effect on insulin-induced activation of PKB. (Kotani et al, 1998). These studies indicate that GLUT4 translocation in these cells is dependent on both atypical PKCs (PKC-λ/ζ) and PKB activation. However, although the insulin signal passes through PI3-kinase, downstream the pathway diverge into 2 independent pathways, a PKB pathway and a PKC-λ pathway, and the latter pathway contributes, at least in part, to insulin stimulated glucose uptake.

4. Insulin mitogenic signaling

In addition to its classical metabolic effects, insulin is a mitogen to many cell types (Lev-Ran, 1998). Some of the pathways described below have been demonstrated to be activated in response to insulin, and others are likely effectors, given their roles in response to other mitogens.

As already described, once activated, the insulin receptor attracts IRS1/2 as well as Shc through their PTB- or PH- domains (Sun et al, 1995; Pronk et al, 1993; White et al, 1985). SH2-containing adapter

proteins bind to phosphorylated IRS1/2 and Shc. One such signaling molecule is the growth factor receptor-binding protein 2 (Grb2). Grb2 is an adapter protein that consists predominantly of one SH2 and two SH3 domains and has no intrinsic catalytic activity (Lowenstein et al, 1992). SH3 domains have specificity for proline-rich sequences (Ceresa and Pessin, 1998). Through one SH3 domain, Grb2 associates with mSos, the mammalian homolog to the Drosophila *son-of-sevenless* protein (Skolnik et al, 1993; Li et al, 1993). mSos is a guanine nucleotide exchange factor (GEF) and promotes the exchange of GDP for GTP on p21Ras (Simon et al, 1991; Sprang, 2001). Conversion to the active GTP bound form of Ras results in activation and the ability to couple with downstream effector molecules (Moodie et al, 1993). GTPase activating proteins (GAPs) promote intrinsic GTP hydrolysis, causing rapid conversion of Ras to the GDP- bound inactive conformation (Cales et al, 1988).

Hyperphosphorylation of Sos on serine/threonine residues causes dissociation of the Grb2-Sos complex (Zhao et al, 1998). Previous studies have suggested that, in addition to the GAPs, this is a second negative feedback loop controlling the Ras activation/inactivation cycle. It was shown *in vitro* that activation of the Raf-MEK-ERK kinase cascade generates this negative feedback signal from MEK (ERK kinase) or a downstream kinase (Langlois et al, 1995). However, activation of Ras, independent of the Grb2/Sos complex has also been illustrated, demonstrating that Grb2 function is not always necessary for Ras activation (Fucini et al, 1999).

4.1 Attachment of Ras to the plasma membrane

Ras is a member of a superfamily of small guanosine triphosphates (GTP-ases) consisting of more than a 100 proteins (See reviews by Symons and Takai, 2001; Reuther and Der, 2000). There are four Ras proteins, H-Ras, N-Ras, K-Ras4A and K-Ras4B. The last two are products of alternative splicing. Ras proteins are highly homologous and 21kDa in size. They share 85% sequence identity, but all 4 proteins are 100% identical in the region important for downstream effector interaction. The only difference in their sequence is in the carboxy-terminal region. This region contains the CAAX-motif responsible for targeting Ras to the plasma membrane. Activation of Ras by GTP-loading is dependent on localization to the plasma membrane (Goalstone and Draznin, 1998).

Inactive Ras is synthesized in the cytoplasm. The CAAX (the C is a cysteine, A an aliphatic amino acid and X is a serine or methionine) tetrapeptide undergoes three post-translational modifications (Gibbs et al, 1997). Firstly, Ras is farnesylated. This process is catalyzed by farnesyl transferase, causing the attachment of a 15-carbon lipid chain (C15 isoprenoid) to the cysteine residue of the CAAX motif (Schaber et al, 1990). After farnesylation, the AAX residues are cleaved by the endopeptidase enzyme from the endoplasmic reticulum. The final step is the methyl esterification of the carboxy-terminal, catalyzed by carboxy-methyl transferase (Clarke et al, 1988). In addition, Ras

must be anchored to the plasma membrane in order to function. Hydrophobicity is conferred on H-Ras, N-Ras and K-Ras4A by palmitoylation of an upstream cysteine. In contrast, K-ras4B has a lysine- rich polybasic region that associates with the phosphate groups in the plasma membrane (Hancock, 1990). These are the final steps necessary for Ras to insert into the inner leaflet of the plasma membrane (Hancock et al, 1991).

4.2 Ras downstream effector pathways

For a long time it was believed that Ras activates a linear kinase cascade, with Raf as the immediate downstream kinase (Fig.III). It has become clear that once activated, Ras can in turn activate a variety of downstream signaling pathways. However, Raf was the first effector identified downstream of Ras (Vojtek et al, 1993).

4.2.1 Raf activation- the main effector pathway

Raf proteins have an amino-terminal regulatory domain and carboxy terminal kinase domain (Kerkhoff and Rapp, 2001). Activation of Raf is very complex and not only requires movement to the membrane and binding to GTP-bound Ras, but also involves the 14-3-3 proteins, heat-shock proteins and multiple phosphorylations (Morrison and Cutler, 1997). Raf proteins are phosphorylated on threonine, tyrosine and serine residues. Raf interaction with Ras is dependent on two domains and binding of Ras to both is required (Drugan et al, 1996). The Ras binding domain, binds Ras first, followed by the cysteine rich domain (Brtva et al, 1995). The cysteine rich domain is involved in the interaction of Raf with lipid molecules and 14-3-3 proteins (Rommel and Hafen, 1998).

It has always been reported that recruitment of Raf to the membrane and subsequent activation is dependent on the Ras/ Raf interaction. However, Raf-1 also has distinct binding domains for two phospholipids, phosphatidic acid and phosphatidylserine (Ghosh et al, 1996). A recent publication indicates that recruitment of Raf is a function of phosphatidic acid, whereas the activation of Raf is because of the interaction between Raf and the two binding domains of activated Ras (Rizzo et al, 2000).

The amino-terminal of Raf negatively regulates its catalytic activity (Cutler et al, 1998; Winkler et al, 1998). There is evidence that Ras binding reduces this inhibitory effect. For Raf to be activated it needs both the Ras signal and phosphorylation on serine and tyrosine residues (Mineo et al, 1997; Marais et al, 1997). Important phosphorylations sites includes tyrosine 340 and 341 and serine 338 and 339 (Mason et al, 1999; Diaz et al, 1997). Phosphorylation of serine 259 results in binding of the scaffolding 14-3-3 proteins (Muslin et al, 1996). Although there is still a lot of controversy on the

exact mechanism of interaction, it is clear that 14-3-3 proteins can affect Raf activity in either a positive or negative manner (Thorson et al, 1998). One theory is that in resting cells, 14-3-3 proteins are bound to Raf at serine 259, keeping it in an inactive conformation (Morrison et al, 1993). Binding of Ras to the Ras binding domain of Raf causes displacement of the 14-3-3 protein, giving Ras access to the cysteine rich domain of Raf. The displaced 14-3-3 protein binds with higher affinity to serine 621 and stabilizes the active Raf (Morrison and Cutler, 1997). The other scenario is that a 14-3-3 dimer keeps Raf in an inactive state. Once localized and activated at the membrane, Raf can associate with protein phosphatase 2A. This interaction destabilizes the 14-3-3 and phosphoserine 259 interaction, the phosphatase removes the phosphate, allowing one arm of the 14-3-3 dimer to interact with upstream activators (Kolch, 2000).

Additional binding proteins for Raf-1 are Hsp90 and Cdc37. The function of the heat shock protein, Hsp90, seems to be stabilization of the tertiary Raf structure (Schulte, 1995). Later it was found that Cdc37 is required for the association of Hsp90 with Raf (Silverstein et al, 1998). The exact role of the involvement of Cdc37 is still not clear.

The three Raf isoforms (Raf-1, A-Raf and B-Raf) share Ras as a common upstream activator and MEK as a downstream substrate (Robinson and Cobb, 1997). Activation of MEK requires phosphorylation of two serine residues in the kinase activation loop (Yan and Templeton, 1994). This kinase is classified as a dual-specificity kinase, since it can phosphorylate both threonine and tyrosine residues (Dhanasekaran and Premkumar, 1998). Of the known MEK family members, MEK 1 and 2 act on ERK1 and 2 (also referred to as p42 and p44 Mitogen-activated protein kinase or MAPK) (Zheng and Guan, 1993). MEK 1/ 2 has a proline rich region in their carboxy-terminal domains that is absent from other family members (Catling et al, 1995). Once activated, MEK1/ 2 activate ERK1/2 through phosphorylation at a threonine and tyrosine in the –Thr-Glu-Tyr- motif (Avruch, 1998). Activated ERKs translocate to the nucleus where they phosphorylate and activate various transcription factors such as Elk-1 and other targets leading primarily to proliferation (Cobb, 1999).

4.2.2 The Ras effector- PI3-kinase

From the literature it is clear that the lipid kinase, PI3-kinase, is the second best characterized downstream effector of Ras (See reviews Bos, 1998; Jun et al, 1999; Rommel and Hafen, 1998). As already described, PI3-kinase is important in insulin metabolic signaling. Binding of PI3-kinase regulatory subunits to activated receptor tyrosine kinases or the IRS docking proteins causes activation of the kinase. However, ample *in vitro* and *in vivo* findings indicate that PI3-kinase can also be activated through direct interaction with Ras-GTP (Kodaki et al, 1994; Rodriguez-Viciana et al, 1994). This leads to activation of the lipid kinase activity of PI3-kinase (Fig.III). The lipid products of PI3-

kinase not only bind to PKB (as discussed in section C3.2.1, page 16), but also to the Rho family GTPase, Rac (Shields et al, 2000). PI3-kinase binds to the Rac GDP/GTP exchange factor, which activates Rac, and this in turn activates nuclear factor-kappa B (NF-κB) (Cammarano and Minden, 2001).

PKB has multiple substrates, which it either activates or inhibits (Khwaja, 1999). Interestingly, although certain substrates are inhibited and others activated, the function of both of these interactions is protection of the cell against apoptosis. An example is phosphorylation by PKB of the proapoptotic protein Bad. The scaffolding protein 14-3-3 binds to the phosphorylated region and this interaction prevents Bad from translocating to the mitochondria where it would bind and inhibit the anti-apoptotic proteins Bcl or Bcl-x_L (Fang et al, 1999). Phosphorylation of the substrate (eg. Bad) creates the ideal binding site for the 14-3-3 proteins. However, binding of the 14-3-3 proteins is not always required for this negative regulation exerted by PKB. PKB can inhibit downstream substrates, such as GSK-3. Inactivation of GSK-3α and β is achieved through phosphorylation of serine 21 and serine 9, respectively (Cross et al, 1995). Substrates that are activated include Ikappa B kinase (IKK) (Ozes et al, 1999). The end result is the release of the NF-κB transcription factor and protection against apoptosis, through stimulation of expression of anti-apoptotic genes (Romashkova and Makarov, 1999).

4.2.2.1. Cross-talk between Raf and PI3-kinase

It has been shown that the Raf-MEK-ERK cascade in growth factor signaling diverges and "cross-talks" with other pathways. In COS cells, PI3-kinase inhibitors suppressed activation of both endogenous ERK2 and Ras by low concentrations of epidermal growth factor (EGF) (Wennstrom and Downward, 1999). They postulated that PI3-kinase-sensitive events may occur both upstream of Ras and between Ras and ERK2. Similarly, in 3T3-L1 adipocytes insulin signaling involves a wortmannin-sensitive PI3-kinase in the interaction between activated Ras and Raf-1 kinase (Suga et al, 1997). Further, in a rat skeletal mucle cell line L6, wortmannin apart from blocking metabolic signaling induced by insulin and insulin-like growth factor-1 at the level of GSK-3, the PI3-kinase inhibitor also prevented the activation of Raf-1 (Cross et al, 1994). All of these results suggest that PI3-kinase can signal to and activate the mitogenic Raf-MEK-ERK cascade (Fig.III).

In contrast, Zimmermann and Moelling (1999) found that PKB can directly phosphorylate Raf on serine 259. The kinase activity of Raf is regulated by phosphorylation of a serine 259 residue. This creates a binding site for 14-3-3 protein, inactivating Raf. This negative regulation of Raf by PKB shifted the biological response in a human breast cancer cell line from growth arrest to proliferation.

This report provided evidence for negative cross-talk between two Ras effectors at the level of Raf and PKB (Zimmermann and Moelling, 1999).

4.2.2.2 Ras effectors regulate the cell cycle

The primary effect of Ras activation in most cell types is proliferation. Mitogenic stimulation causes cells to exit the G0 phase or resting state, enter the G1 phase, followed by DNA synthesis (S-phase). Positive regulators involved are the G1 cyclin-dependent kinases (CDKs). CDK4 and CDK6 complex with the D-type cyclins (D1, D2, D3) and cyclin E complexes with CDK2 (Dulic et al, 1992; Marshall, 1999). Activation of the cyclinD-Cdk4/6 and cyclinE-Cdk2 complexes promotes phosphorylation of the retinoblastoma tumor suppressor protein (Ezhevsky et al, 2001). This protein is associated with the transcription factor E2F and represses its activity (Lundberg and Weinberg, 1998). Hyperphosphorylation of the retinoblastoma protein, promotes the release of E2F and transactivation of genes necessary for S phase entry (Dynlacht et al, 1994; Suzuki-Takahashi et al, 1995). Negative regulators are the CDK inhibitors (CKIs) and include the INK4 family ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$) (Hirai et al, 1995) and the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) (Sherr and Roberts, 1999).

Activation of Ras is required for resting cells to enter the cell cycle at G1 (Peeper et al, 1997). For cells to enter DNA synthesis, endogenous Ras function is required throughout most of G1. Upon mitogen stimulation of resting cells (G0), two peaks of Ras activation are present, the first immediately when the cells enter G1 and the second in mid-G1 (Taylor and Shalloway, 1996). The first peak is associated with the Raf-MEK-ERK pathway and the second with PI3-kinase-PKB activation (Gille and Downward, 1999).

A relationship between Ras signaling activity and the regulation of cyclin D1 and the CDK inhibitors has also been established. Activation of Ras is required for mitogen-stimulated up-regulation of cyclin D1 and $p21^{Cip1}$ and down-regulation of $p27^{Kip1}$ protein expression. Ras activation can control both positive (cyclin D1) and negative ($p21^{Cip1}$ and $p27^{Kip1}$) regulators required for G0 exit, G1 progression and proliferation (Pruitt and Der, 2001). Ras upregulation of cyclin D1 is dependent mainly on activation of the Raf/MEK/ERK kinase cascade. For example, activation of Raf is associated with increased cyclin D1 protein levels and repression of the $p27^{kip1}$ cyclin-dependent kinase inhibitor (Kerkhoff and Rapp, 1997).

It is clear that once activated, Ras can activate different downstream effector pathways. Also, many growth factor receptor pathways activate both the Ras/Raf and PI3-kinase signaling cascades (Yu and Sato, 1999). Insulin stimulated mitogenic signaling has been reported to be mostly dependent on the

Raf/MEK/ERK pathway (Xi et al, 1997). In some cells, however, insulin appears to mediate proliferation primarily through a PI3-kinase-dependent, but ERK-independent pathway. In primary hepatocytes (Band et al, 1999) and 3T3-L1 adipocytes (Cheatham et al, 1994) insulin activation of proliferation has been shown to be largely ERK-independent, and instead involves PI3-kinase and $p70^{s6k}$. The latter is a 70kDa serine/threonine kinase, stimulated in response to insulin and a possible downstream target of PI3-kinase. It is therefore clear that growth factors such as insulin can stimulate cell proliferation through multiple alternative routes, and that in osteoblasts, ERK and PI3-kinase are likely to be central effectors.

D. AIMS OF THIS STUDY

There is a clear lack of effective anabolic agents for bone. It would therefore be helpful to explore the exact signaling pathways used by different mitogens in bone-building osteoblasts. Insulin appears to play a key role as an osteoblast mitogen in both developing and adult bones. We therefore set out to examine how insulin and other growth factors signal to osteoblast proliferation, how these pathways are negatively affected by bone damaging glucocorticoids, and whether the insulin mimetic, vanadate, via its inhibition of protein tyrosine phosphatase activity, would effectively combat GC damage.

CHAPTER 2

EXPERIMENTAL PROCEDURES

1. Materials

Polyclonal antibody against phosphorylated ERK (threonine and tyrosine phosphorylated, p42 and p44) and phospho-Akt/PKB antibody were from New England Biolabs, Inc. (Beverly, MA). Polyclonal antibodies against rabbit SHP-1 (100 μg/ml), ERK (100 μg/ml), MEK (100 μg/ml), Raf-1 (100 μg/ml) and polyclonal goat PTP-1B (200 μg/ml) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal rabbit phospho-tyrosine antibody was from Upstate Biochemicals Incorporated (Lake Placid, NY). Reagents for ECL chemiluminescent detection, secondary peroxidase-coupled anti-rabbit antibody from donkey, and [*methyl*-^{3}H] thymidine were from Amersham Pharmacia Biotech (UK). Immunopure protein A agarose beads were purchased from Pierce Scientific (Rockford, Illinois). Immobilon-P transfer membrane was from the Millipore Corporation (Bedford, MA). Liquid scintillation cocktail and poly-Q vials were from Beckman Coulter. U0126 was purchased from Promega (Madison, WI). FCS was from Delta Bioproducts (Johannesburg, R.S.A). All other chemicals including tissue culture media, O-tetradecanoylphorbol 13-acetate (TPA), dexamethasone, wortmannin and insulin were of the highest grade, obtained from Sigma Chemical Co. (St. Louis, MO).

2. Cell treatments

U0126 and Wortmannin were dissolved in DMSO to 10 mM and 2 mM, respectively. If inhibitors were added to cells, control cultures were treated with equivalent amounts of DMSO. Insulin was dissolved in acidified water, pH=3, immediately before use. Dexamethasone and TPA were dissolved in ethanol and DMSO, respectively. Aliquots were stored at -20°C. Sodium orthovanadate was dissolved in sterile water immediately before use.

3. Tissue Culture

MBA-15.4 and MBA-15.6 mouse bone marrow stromal cells (donated by Professor S. Wientroub, Tel Aviv University, Israel) were cultured in Dulbecco's modified Eagle's medium (DMEM). UMR-106 rat cells (donated by Prof TJ Martin, Australia) were grown in Minimum essential Eagle's medium (MEM) and the MG-63 human preosteoblasts in MEM alpha modification. All media were bicarbonate buffered and supplemented with 10% heat-inactivated FCS (unless serum-starved),

100U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells were lifted by scraping with a rubber policeman, before being seeded in 24-well plates (proliferation studies), or 10cm culture dishes (immunoprecipitation), except for the UMR-106 cells that were washed once with phosphate-buffered saline (PBS) and trypsinized with 0.05% trypsin in 0.01% EDTA. Details of treatments are provided in the figure legends.

4. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western blots

Serum-starved cells in 10cm culture dishes were treated as described, washed twice with ice-cold wash buffer (20 mMTris, pH7.5; 100 μM Na_3VO_4; 50 mM NaF; 1 mM EGTA; 1 mM levamisole; 1 mM PMSF) and sonicated on ice in 400 μl of cold lysis buffer (wash buffer containing 1 μg/ml each of aprotinin, leupeptin and pepstatin). Cell lysates were centrifuged at 4°C (14,000 x g for 8 min) and protein concentrations were determined using the Bradford method, with bovine serum albumin as a standard (Bradford, 1976). Equal-protein samples were diluted in Laemmli sample buffer containing β-mercaptoethanol and boiled for 5 min (Laemmli, 1970). Proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred by electroblotting to Immobilon-P membrane (Millipore Corp.). Membranes were immersed in methanol for 10 sec, allowed to air dry for 15 min and blocked in Tris-buffered saline (TBS: 20 mM Tris, pH7.5; 137 mM NaCl) containing 0.1%Tween-20 (TBS-T) and 5% nonfat dry milk for 1 h at room temperature. Blots were washed 5 times for 5 min with TBS-T and incubated overnight at 4°C with rabbit polyclonal ERK antibodies against p42/p44 (Santa Cruz Biotechnology), SHP-1, Raf-1, MEK, phospho-42/44 ERK (New England Biolabs) or phospho-Akt/PKB (New England Biolabs). The membranes were rinsed in TBS-T followed by a 1 h incubation at room temperature with the secondary antibody [anti-rabbit IgG horseradish peroxidase (from donkey)]. The membranes were washed in TBS-T and the transferred proteins were detected using chemiluminescence (ECL system, Amersham Pharmacia Biotech).

5. Cell proliferation

Serum-starved cells sub-cultured into 24-well plates were treated with various agents and for time periods as described in the figure legends. In every experiment 2μCi/well [^{3}H] thymidine (Amersham Pharmacia Biotech) was added to the medium for the last 2 h of incubation. The plates were placed on ice, washed twice with cold PBS before being quick-frozen at -80°C for 30 min. Cells were thawed at room temperature and lysed in 0.1 M NaOH-0.1% SDS before adding 50% trichloroacetic acid (TCA) for an overnight precipitation at 4°C. The pellet was washed once with cold 10% TCA, dissolved in 0.1 M NaOH and counted in Ready Gel aqueous scintillation fluid (Beckman) to obtain a measurement of [^{3}H] thymidine incorporation during DNA synthesis.

6. Immunoprecipitation

Serum-starved cells in 24-well plates were treated as indicated, washed twice with ice-cold wash buffer (20 mMTris, pH7.5; 100 μM Na_3VO_4 ; 50 mM NaF; 1 mM EGTA; 1 mM levamisole; 1 mM PMSF), collected by scraping with a rubber policeman, and sonicated on ice in 400 μl cold lysis buffer (wash buffer containing 1 μg/ml each of aprotinin, leupeptin and pepstatin). Supernatants clarified by centrifugation were incubated overnight at 4°C with 4 μg of the relevant antibody/ml lysate. Immune complexes were collected by incubation for 2 h at 4°C with 100 μl protein A agarose bead slurry. The beads were washed three times with ice-cold PBS, solubilized in 50 μl Laemmli sample buffer, boiled for 5 min and the proteins separated by SDS-PAGE, followed by Western blotting.

7. Statistical analysis

Results are given as the mean ± S.E.M or ± SD as indicated. Data were analyzed using GraphPad Prism v 2.01 (GraphPad Software, Inc.) by one-way Anova with Dunnett's Post-hoc test or one-way Anova with Bonferroni Post-hoc for multi-group comparisons. Differences were considered statistically significant at $P<0.05$.

CHAPTER 3

RESULTS

1. Insulin-induced proliferation under different serum conditions and in different osteoblast cell lines

The mitogenic phorbol ester TPA (1 μg/ml), and 20% FCS which is a cocktail of growth factors, are known mitogenic stimuli for most osteoblast cell lines. These mitogens were included in Fig.1A and B as positive controls. To determine the effect of insulin as an osteoblast mitogen, 50% confluent cells were serum starved in 1% FCS for 24 h, prior to treatment with increasing doses of insulin (0.01-10 μg/ml) for a further 24 h. DNA synthesis was measured by [^{3}H] thymidine incorporation. Although a clear dose response was not observed in all experiments, an increase in proliferation of ~70% above control levels was seen with all 4 doses of insulin used (Fig.1A). However, this was highly variable from experiment to experiment. This experiment was repeated, but instead of using 1% FCS, the cells were serum starved in 0.5% FCS for 24 h prior to insulin treatment to lower the proliferation rate of controls. As shown in Fig.1B, there was an increase in proliferation, which was maximal at 10 μg/ml, causing an increase of ~60% above control cells. It was not possible to maintain live cultures in the complete absence of growth factor-containing serum. By lowering the serum to 0.5%, the control thymidine incorporation was much lower and the percent increase in proliferation above control levels was more consistent in later experiments.

To compare insulin response in a variety of osteoblast cell lines, a relatively immature MG-63 human and the differentiated UMR-106 rat osteoblast cell line were treated with the same insulin concentrations (0.01-20 μg/ml). The cells were serum starved in 0.5% FCS for 24 h, followed by insulin treatment for 24 h. Insulin stimulated proliferation in the MG-63 human cell line (Fig.1C) in a concentration-dependent manner with the maximal increase (160%) obtained with the top three doses 1,10 and 20 μg/ml of insulin. The mature UMR-106 rat osteoblasts responded with less sensitivity to insulin, producing a more modest stimulation (6-21%) of cell proliferation (Fig1.D) with 0.1-20 μg/ml of insulin treatment when compared with controls. Based on this data, in subsequent experiments insulin was used at a concentration of 10 μg/ml. The younger osteoblasts (mouse MBA-15.4 and human MG-63) were much more insulin sensitive, compared to the more mature UMR-rat cells. Although we did not have an immature rat cell line to test, we concluded that differentiation state was important when looking at the effect of insulin on osteoblast proliferation.

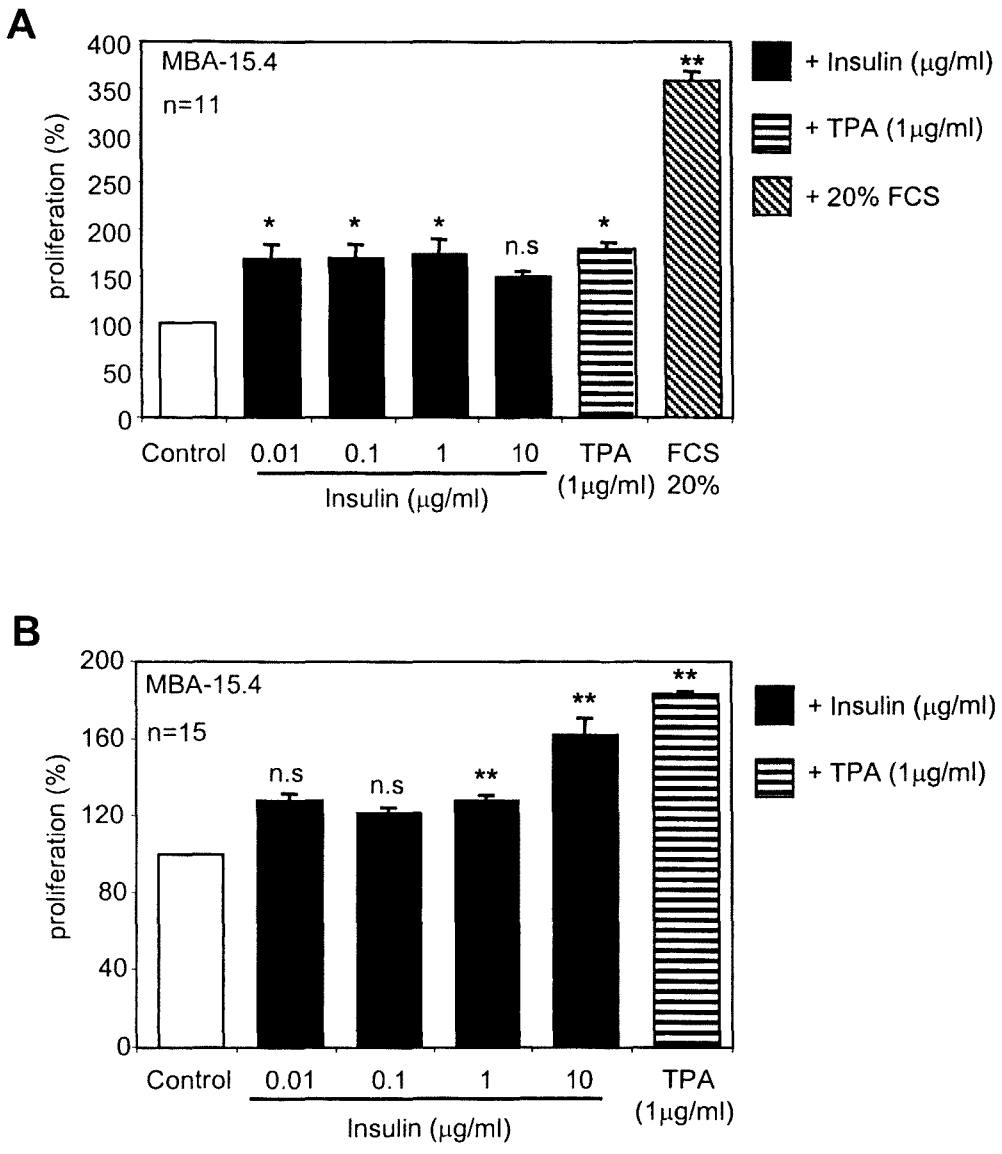

FIG.1. **Insulin activation of proliferation in mouse preosteoblasts.** Dose-response of (*A, B*) MBA-15.4 mouse cell line to insulin treatment. Cells were cultured to 50% confluency and starved overnight in medium containing 1% FCS (*A*) or 0.5% FCS (*B*). This was followed by treatment with different concentrations of insulin as indicated for 24 h. TPA (1 μg/ml) and FCS (20%) were included as positive controls in *A* and *B* only. DNA synthesis was measured by incorporation of [^{3}H] thymidine. Results shown are the mean ± S.E.M of eleven (*A*) and fifteen (*B*) independent experiments expressed as percent of respective controls. *P< 0.05, **P< 0.01 compared with control; n.s: not significant compared with control (*One-way Anova with Dunnett's post-hoc*).

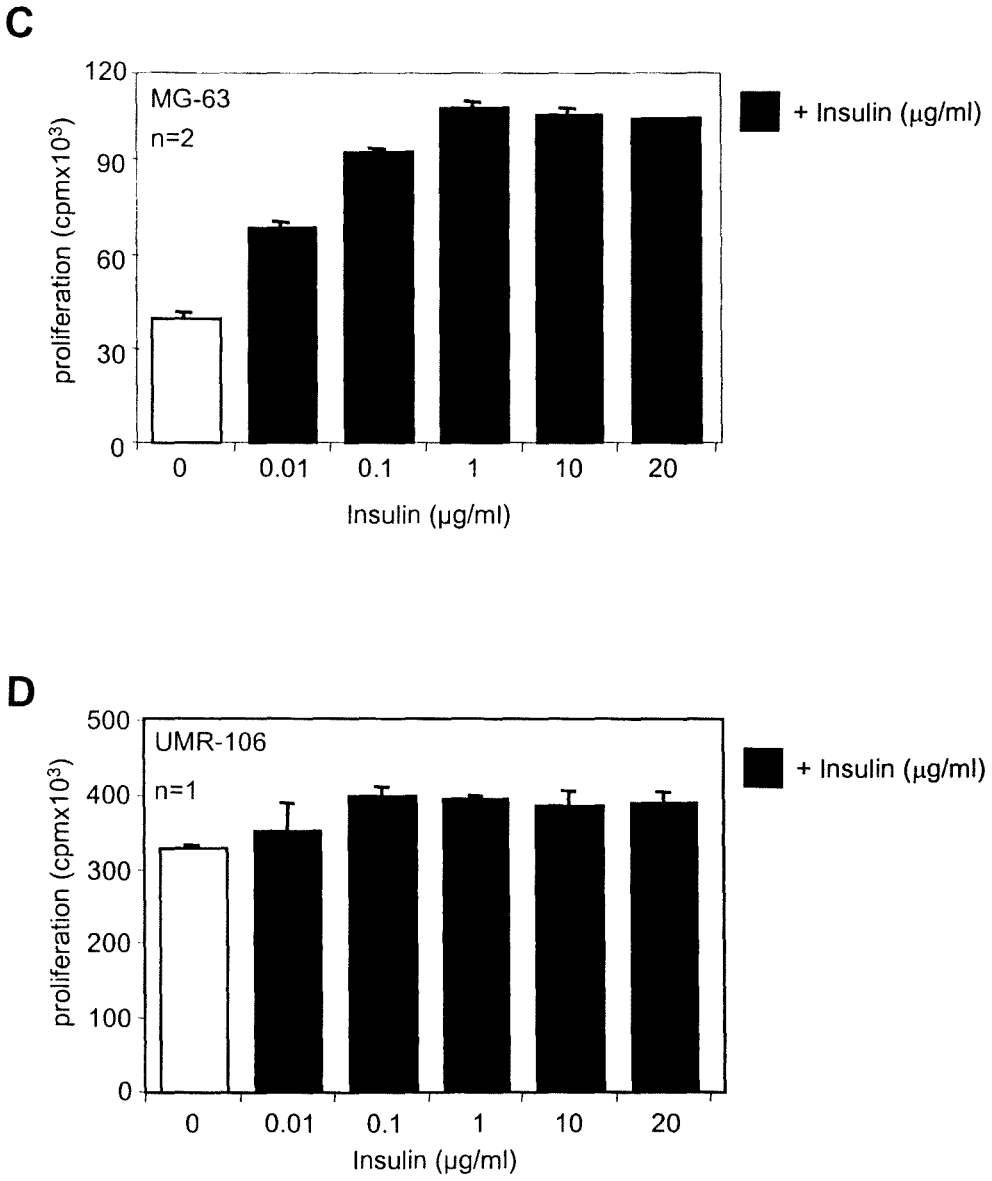

FIG.1 (cont.). **Insulin activation of proliferation in human and rat osteoblast cell lines.** Dose-response of (*C*) MG-63 human and (*D*) UMR-106 rat cell lines to insulin treatment. Cells were cultured to 50% confluency and starved overnight in medium containing 0.5% FCS (*C, D*). This was followed by treatment with different concentrations of insulin as indicated for 24 h. DNA synthesis was measured by incorporation of [^{3}H] thymidine. Results shown are representative experiments performed in triplicate. Fig.1C and D is courtesy of Dr Y Engelbrecht.

2. ERK activity was induced by insulin, TPA and 20% FCS treatment in MBA-15.4 preosteoblasts

In order to evaluate the ability of insulin to activate ERK, relative to the known potent ERK activators, TPA and FCS, serum starved MBA-15.4 cells were stimulated with optimal concentrations of mitogens, 10 μg/ml insulin, 100 ng/ml TPA or 20% FCS for various periods of time. Phosphorylation of ERK was assessed by Western blots, using an antibody directed against dual-phosphorylated, active ERK-1/2 (Fig.2A). Maximum phosphorylation of ERK was seen at the 5 min time point for all 3 mitogens. Insulin produced a more modest stimulation of ERK activity compared to TPA and 20% FCS (80-fold vs 160-fold), and this was greatly attenuated by 60 min, whereas response to the other mitogens remained strong. The total amount of ERK in each lane was similar, as indicated by immunoblot analysis using an antibody to total ERK, and the fold increase in ERK phosphorylation shown in Fig.2B was corrected to reflect active-ERK per equal ERK sample.

3. Effect of inhibition of MEK and PI3-kinase on insulin- and FCS-induced ERK activity

Next we investigated which signaling pathway activated by insulin (metabolic-, mitogenic- or other pathway) mediates ERK activation in MBA-15.4 osteoblasts. As a positive control, experiments were repeated with 20% FCS. The cells were pre-incubated for 30 min with a single treatment of either 10 μM U0126, a specific inhibitor of MEK-1 and -2, or 100 nM wortmannin, a PI3-kinase inhibitor, followed by short (5 and 30 min) or longer (24 h) 10 μg/ml insulin or 20% FCS stimulations. At 10 μM, U0126 completely inhibited insulin-induced phosphorylation of ERK-1/2 at 5 min and at 30 min (Fig.3A). FCS-induced ERK phosphorylation was inhibited by 70% at 5 min and at 30 min of mitogenic stimulation (Fig.3C). After 24 h, U0126 was still inhibitory, reducing insulin- and FCS-stimulated ERK activity by ~90% (Fig.3B) and ~70% (Fig.3D), respectively. Since activation of the PI3-kinase pathway is associated with proliferative signaling by Ras, we also investigated whether this pathway was involved. Pretreatment for 30 min with 100 nM wortmannin decreased ERK activity by 60% after 5 min and almost completely after 30 min of insulin treatment (Fig.3A). Twenty-four hours later, insulin-stimulated ERK activity was still 42% lower in wortmannin treated samples, than in insulin-stimulated samples that received no wortmannin (Fig.3B). However, wortmannin did not significantly affect FCS-stimulated ERK activity at the shorter time periods (Fig.3C), or inhibited ERK activity more slowly, since a 46% reduction in ERK activity was seen 24 h later. This result indicates that activation of ERK in response to insulin and 20% FCS is strongly MEK dependent. A wortmannin sensitive PI3-kinase pathway was clearly involved in ERK response to insulin stimulation at both shorter and longer time periods, whereas wortmannin only visibly inhibited FCS-induced activation of ERK after 24 h.

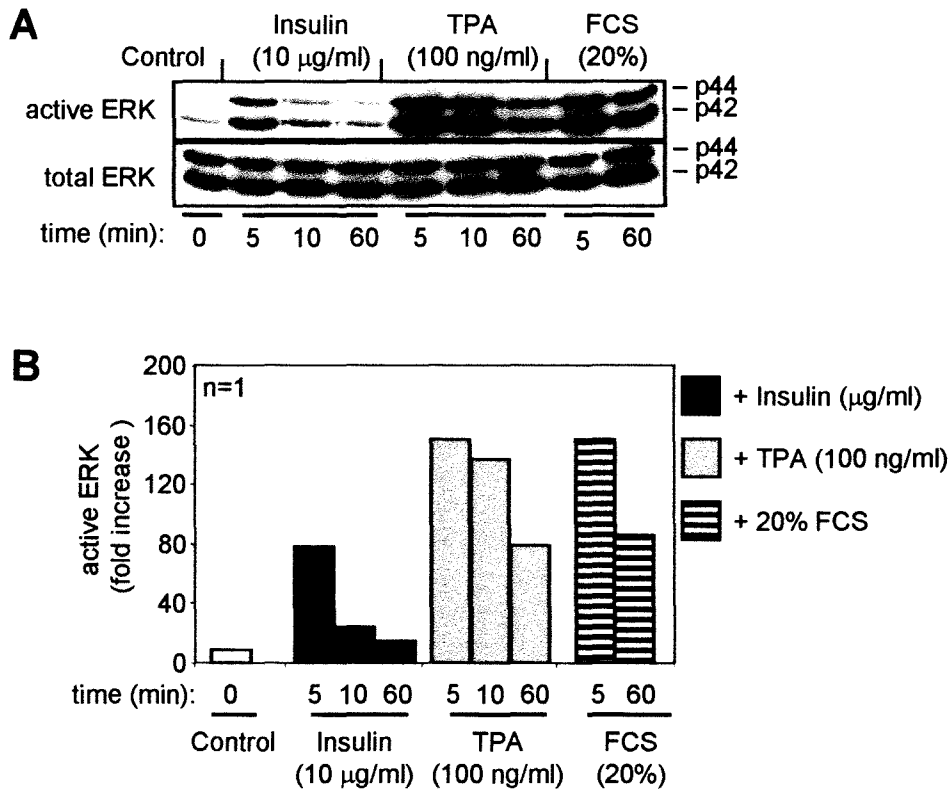

FIG.2. **Activation of ERK-1/2 by various mitogenic stimuli**. Subconfluent MBA-15.4 cells were cultured overnight in medium containing 1% FCS. Cells were treated with 10 μg/ml insulin, 100 ng/ml TPA or 20% FCS for 5, 10 or 60 min, respectively. In order to measure ERK activity of the samples, cells were harvested and lysed and active or total ERKs in equivalent concentrations of total lysate protein were visualized by Western blot analysis using an anti-active ERKp42/p44-specific antibody or a p42/p44 ERK antibody (*A, representative blot*). *B*, represents relative active ERK levels quantified by densitometry of immunoblots. Relative active ERK levels in control cells (no treatment) was given a value of 1.

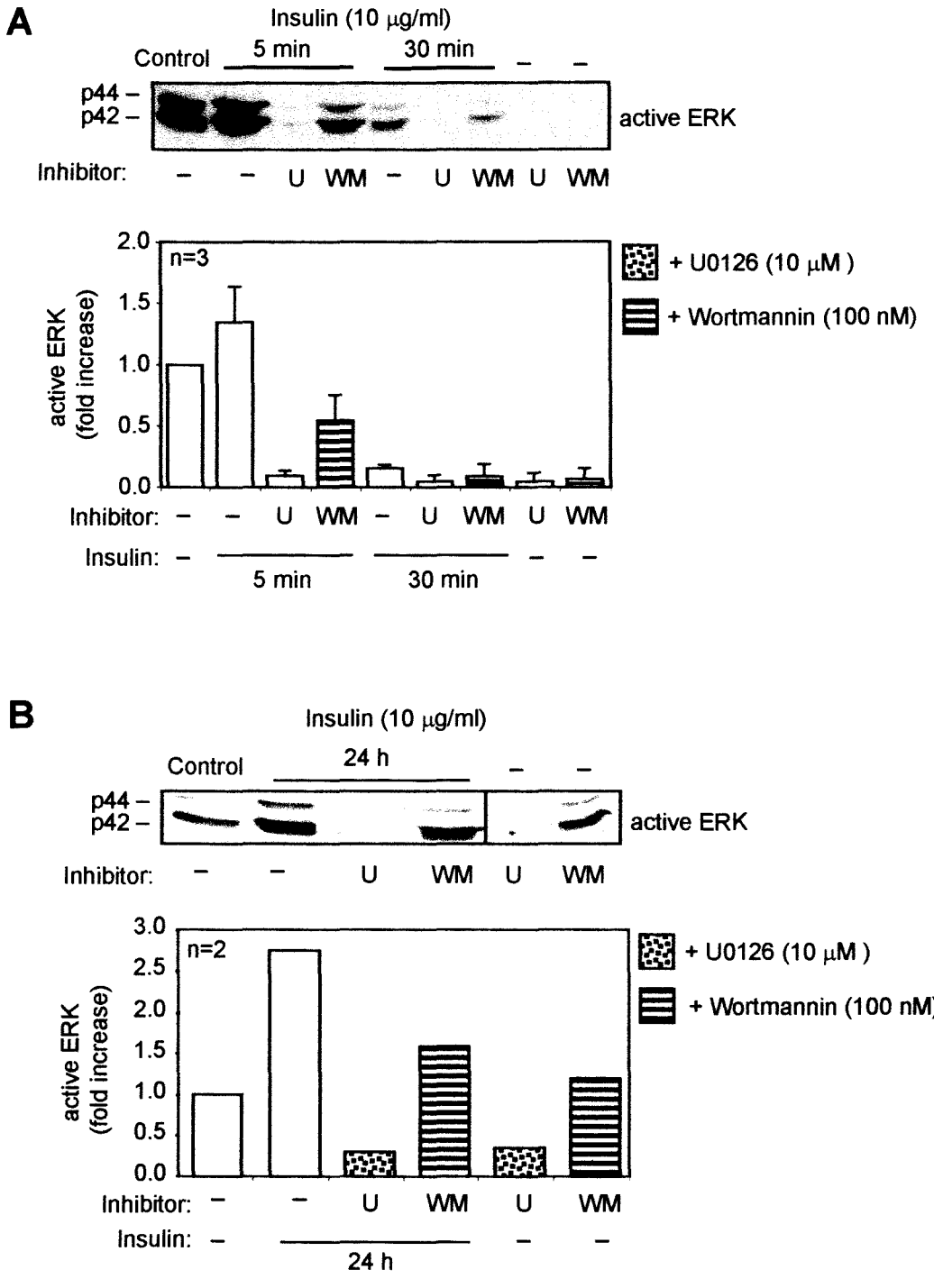

FIG.3. **Inhibition of insulin-induced ERK activation by U0126, a MEK inhibitor compared to partial impairment of ERK activity by wortmannin, a PI3-kinase inhibitor.** Subconfluent MBA-15.4 cells were cultured overnight in medium containing 1% FCS. The cells were pre-incubated for 30 min in the presence or absence of 10 μM U0126 or 100 nM Wortmannin prior to 10 μg/ml insulin (*A,B*) treatment for 5 and 30 min (*A*) and 24 h (*B*), respectively. Cells were harvested and lysed and ERK activity was visualized by Western blot analysis using an active ERKp42/p44-specific antibody. In the *top panels* (*A, B*) a representative Western blot is shown. The *bottom panels* represents averaged data of relative active ERKp42/p44 levels of three (*A*) and one (*B*) independent experiment(s) quantified by densitometry. Relative active ERK levels in control cells (no treatment) was given a value of 1.

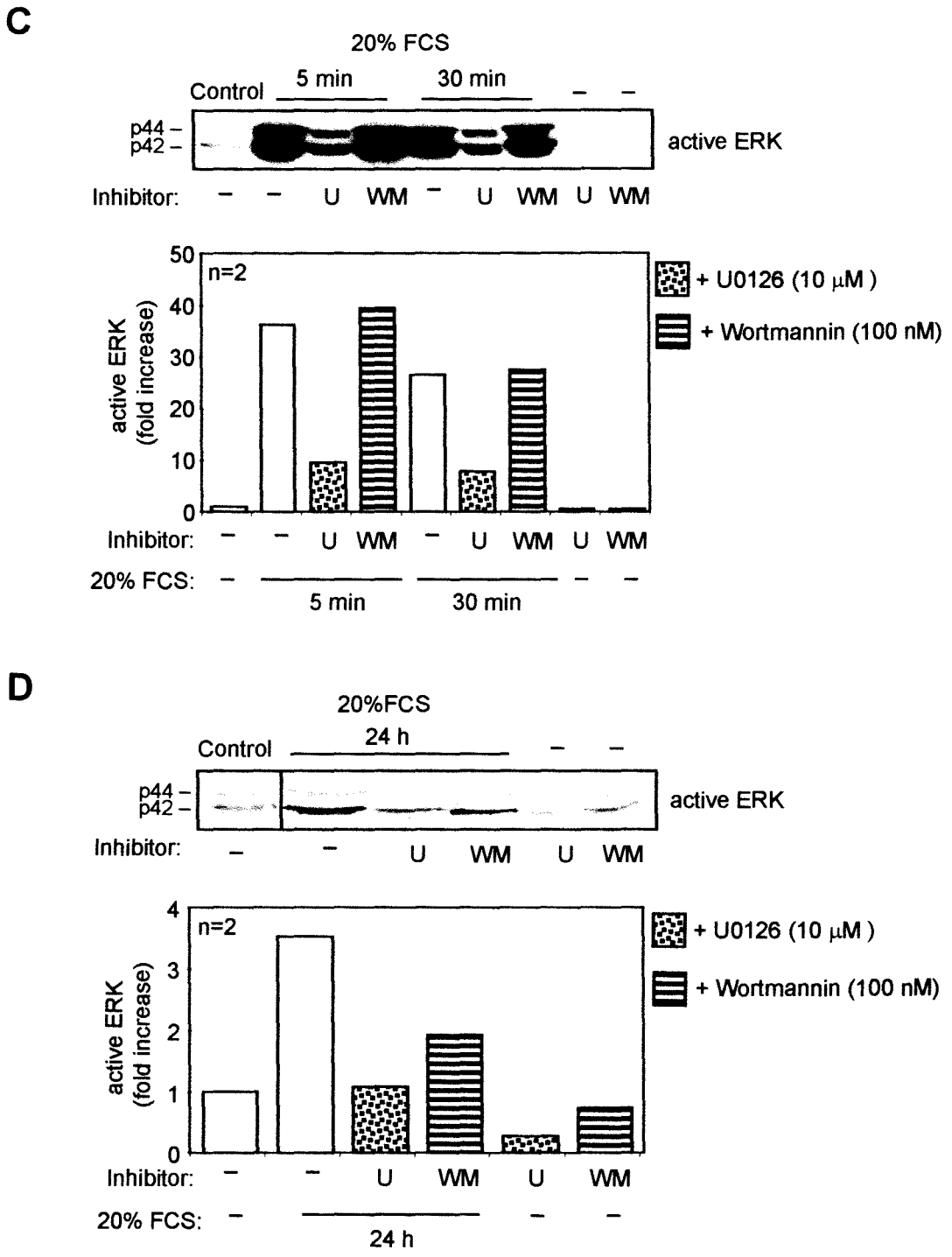

FIG.3 (cont.). **Inhibition of 20% FCS-induced ERK activation by U0126, a MEK inhibitor compared to no or partial impairment of ERK activity by wortmannin, a PI3-kinase inhibitor.** Subconfluent MBA-15.4 cells were cultured overnight in medium containing 1% FCS. The cells were pre-incubated for 30 min in the presence or absence of 10 μM U0126 or 100nM Wortmannin prior to 20% FCS (*C,D*) treatment for 5 and 30 min (*C*) and 24 h (*D*), respectively. Cells were harvested and lysed and ERK activity was visualized by Western blot analysis using an active ERKp42/p44-specific antibody. In the *top panels* (C,*D*) a representative Western blot is shown. The *bottom panels* represents averaged data of relative active ERKp42/p44 levels of one (C,*D*) independent experiment quantified by densitometry. Relative active ERK levels in control cells (no treatment) was given a value of 1.

4. Akt/PKB activation by insulin is PI3-kinase-dependent and wortmannin sensitive

To ensure that under the experimental conditions used, wortmannin did indeed inhibit PI3-kinase, activation of Akt/PKB by PI3-kinase was examined in the presence or absence of wortmannin. Western blots were then performed using an antibody directed against phosphorylated, active Akt/PKB. MBA-15.4 cells were treated for various lengths of time with 10 μg/ml insulin, following 30 min pretreatment with 100 nM wortmannin. At 10 μg/ml, insulin induced a rapid activation of Akt/PKB with a peak occuring at 30 min (Fig.4A). Akt/PKB activation was still detectable at 24 h (Fig.4B). As shown in Fig.4A and B, Akt/PKB phosphorylation by insulin was dramatically reduced by wortmannin at 5, 10 and 30 min and even after 24 h. This confirms that PI3-kinase is an upstream mediator of insulin- induced Akt/PKB activation in MBA-15.4 preosteoblasts, and that we were using wortmannin at the correct concentration and for a suitable time.

5. FCS-stimulated proliferation is MEK-dependent and PI3-kinase-independent, whereas insulin-stimulated cell proliferation is MEK- and PI3-kinase- independent in mouse preosteoblasts

Inhibitors like U0126 and wortmannin are able to block various aspects of insulin action. Given the effect of the two inhibitors on ERK activity (Fig.3), we next studied the effect of U0126 and wortmannin on proliferation stimulated by insulin and 20% FCS in MBA-15.4 cells. Wortmannin, the PI3-kinase inhibitor had no effect on insulin- or 20% FCS- induced proliferation (Fig.5A and B). As expected, the MEK inhibitor, U0126, strongly decreased proliferation in response to 20% FCS. In sharp contrast, insulin-stimulated activation of proliferation was unaffected by MEK inhibition, and in fact, in many experiments, an apparent increase was observed (Fig.5A). This increase was not statistically significant in the small number of repeats presented here, but is interesting because it suggests the existence of a negative feedback mechanism and therefore warrants further investigation. This result raised the question of possible novel pathways being involved in insulin signaling in osteoblasts. To investigate this further, a combination of the two inhibitors was used, blocking both MEK- and PI3-kinase- mediated signals (Fig.5B). Insulin stimulated proliferation was completely unaffected by MEK inhibition or PI3-kinase inhibition or (*diagonal hatched bars*) a combination of the two. In contrast, U0126 inhibited FCS-stimulated proliferation by ~50%. Wortmannin had no effect alone or in combination with U1026 (*diagonal hatched bars*). It is therefore evident that insulin-stimulated proliferation is not dependent on ERK or PI3-kinase signaling, while proliferation in response to 20% FCS is completely MEK-dependent (See Fig.13 in discussion) in the mouse MBA-15.4 preosteoblast cell line.

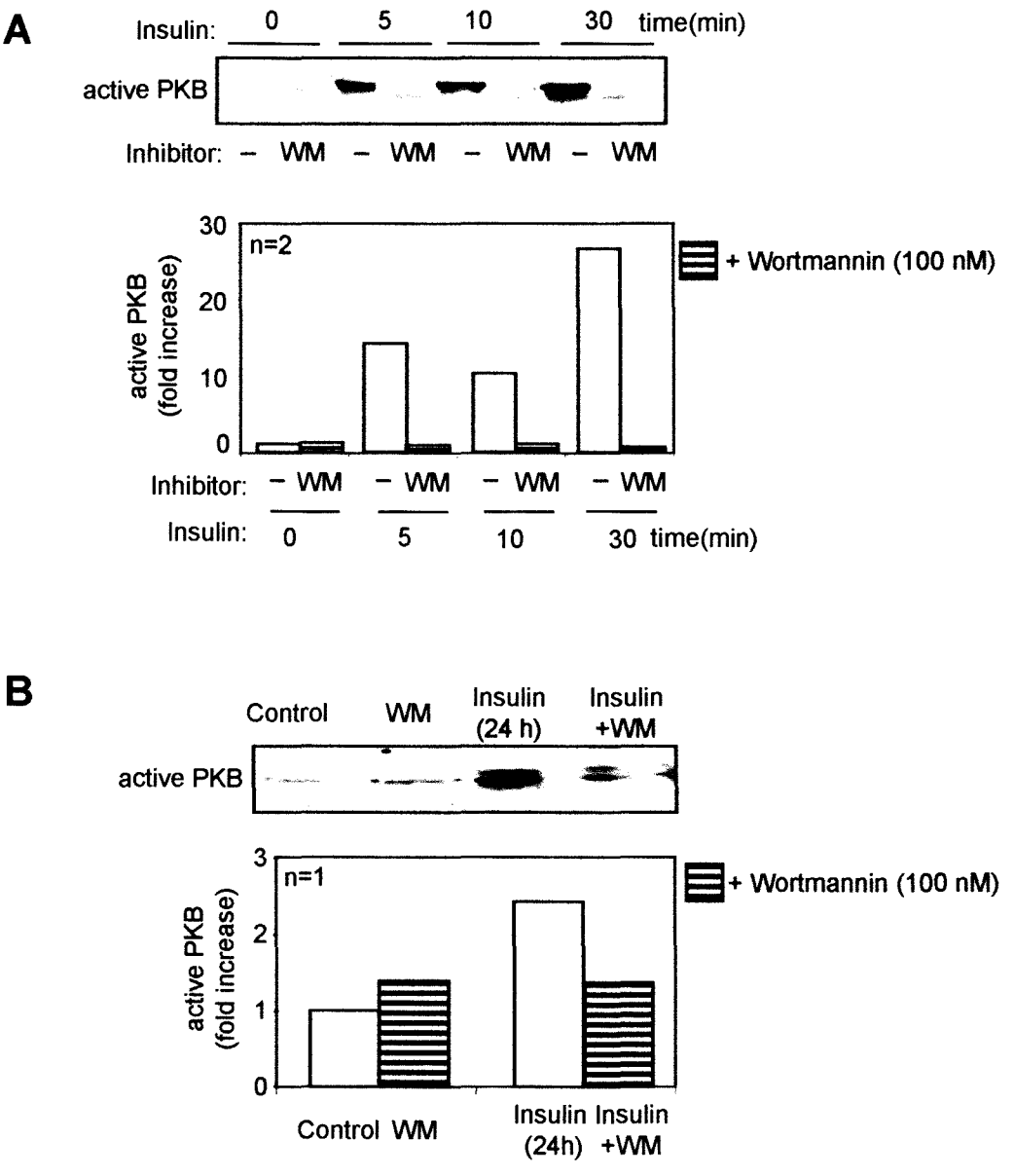

FIG.4. **Inhibition of insulin-induced Akt/PKB activation by wortmannin, a PI3-kinase inhibitor.** Subconfluent MBA-15.4 cells were cultured overnight in medium containing 1% FCS. Cells were pre-treated for 30 min with or without 100 nM wortmannin (*A, B*) for 30 min followed by insulin treatment for various lengths of time i.e. 0, 5, 10 or 30 min (*A*) or 24 h (*B*). Cells were harvested and lysed and active Akt/PKB was visualized by Western blot analysis using an active Akt/PKB-specific antibody. In the *top panels* (*A-B*), a representative Western blot is shown. The *bottom panels* represent relative Akt/PKB levels quantified by densitometry of immunoblots. Relative Akt /PKB levels observed in cells at time 0 was defined as 1 (control).

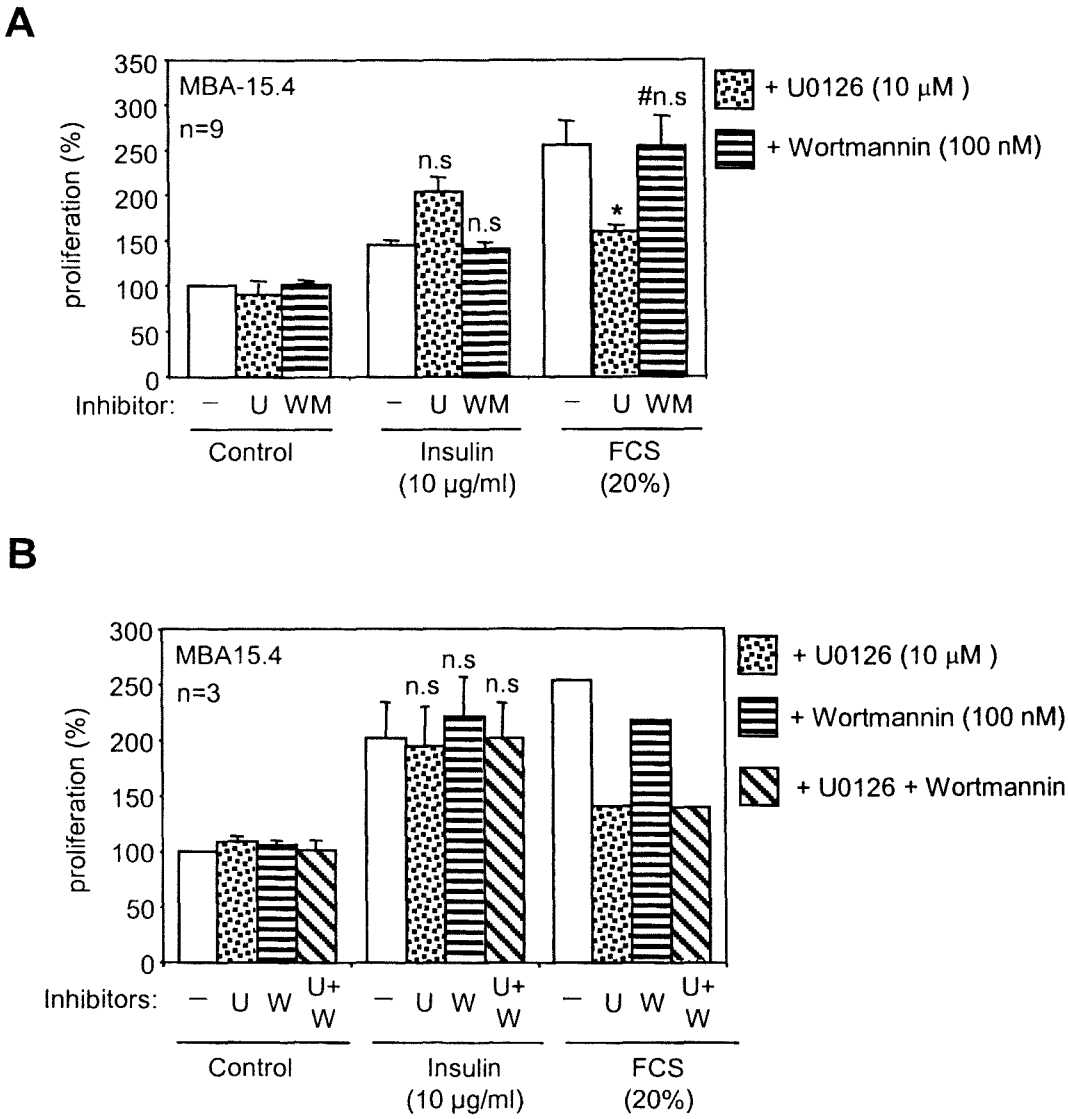

FIG.5. **MEK inhibition by U0126 blocks 20% FCS but not insulin-induced proliferation, while no alterations in proliferation by the PI3-kinase inhibitor, wortmannin were observed.** 50% confluent cells were cultured overnight in medium containing 0.5% FCS. Cells were pre-cultured in the presence or absence of 10 μM U0126 or 100 nM wortmannin alone (*A,B*) or a combination of the two inhibitors (*B, diagonal hatched bars*), prior to a further 24 h treatment without (control) or with 10 μg/ml insulin or 20% FCS (*A,B*). DNA synthesis was measured by [^{3}H] thymidine incorporation. Results represent the mean ± S.E.M. of nine (*A*) and three (*B*) independent experiments and are expressed as percent of respective control. In (*B*) treatment with FCS was performed in one experiment. (*A*), n.s: not significant compared with insulin, #n.s: not siginificant compared with 20% FCS, *P<0.05 compared with 20% FCS. (*B*), n.s: not significant compared with insulin (*One-way Anova with Bonferroni post-hoc for multi-group comparison*).

6. Insulin mediated proliferative signaling was independent of cell maturity, but was different in mouse and human osteoblasts

Since the concentration of U0126, effective for inhibition of ERK activity, did not inhibit insulin-stimulated proliferation, we investigated two higher doses. MBA-15.4 (Fig.6A) and MBA-15.6 (Fig.6B) mouse osteoblasts were stimulated with 20% FCS or 10 μg/ml insulin after pretreatment with increasing doses of U0126 (10-30μM). Proliferation was measured by [^{3}H] thymidine incorporation. In the mouse, MBA-15.4 cells, pre-incubation with U0126 completely inhibited the mitogenic effect of 20% FCS in a dose dependent-manner (*horizontal hatched bars*), without any effect on insulin-mediated proliferation (Fig.6A, *black bars*). To investigate whether this was age and stage dependent, we compared the relatively immature MBA-15.4 osteoblast cell line (Fig.6A) with the more differentiated MBA-15.6 mouse osteoblast cell line (Fig.6B). U0126 again had no effect at all on insulin-stimulated proliferation, while markedly inhibiting FCS-induced proliferation. To determine whether this finding was species-specific, a U0126 dose-response curve for inhibition of insulin-induced proliferation was performed in the MG-63 human osteoblast cell line (Fig.6C). At 10, 20 and 30μM concentrations, U0126 did inhibit insulin-induced proliferation in the human osteoblast cell line. While not obviously toxic to the cells, the inhibitor also reduced basal DNA synthesis. These results suggest that there may be species differences in insulin mitogenic signaling.

7. Insulin mitogenic signaling in human osteoblasts is dependent on ERK and PI3-kinase

Although U0126 did inhibit human osteoblast proliferation in response to insulin, this inhibition was not as severe as controls at all three doses tested. We therefore suspected that insulin signaling in the MG-63 cell line might be less ERK-dependent than responses to other mitogens. We compared proliferation in response to 10 μg/ml insulin versus 100 ng/ml TPA in the absence or presence of 10 μM U0126. Inhibition of MEK with U0126 almost completely abolished TPA-stimulated proliferation and also severely inhibited baseline growth in 0.5% serum (Fig.7A). However, insulin-stimulated proliferation was only inhibited by half, indicating the possible involvement of a second pathway. Alternatively, there may be a drug effect of U0126 which accounts for the apparent insulin decrease, while the 0.5% FCS and TPA inhibition is due to both a drug effect and MEK inhibition. TPA was a stronger mitogen than insulin, stimulating proliferation to 210% of control levels, compared with 170% stimulation by insulin. We therefore tested the involvement of PI3-kinase by inhibiting it with 100 nM wortmannin alone or in combination with 10 μM U0126 (Fig.7B). As before, insulin-stimulated proliferation was only partially inhibited by U0126. Wortmannin alone had a similar effect. However, in combination, U0126 and wortmannin completely abolished cell proliferation, suggesting that the MEK and PI3-kinase pathways separately and additively account for human osteoblast

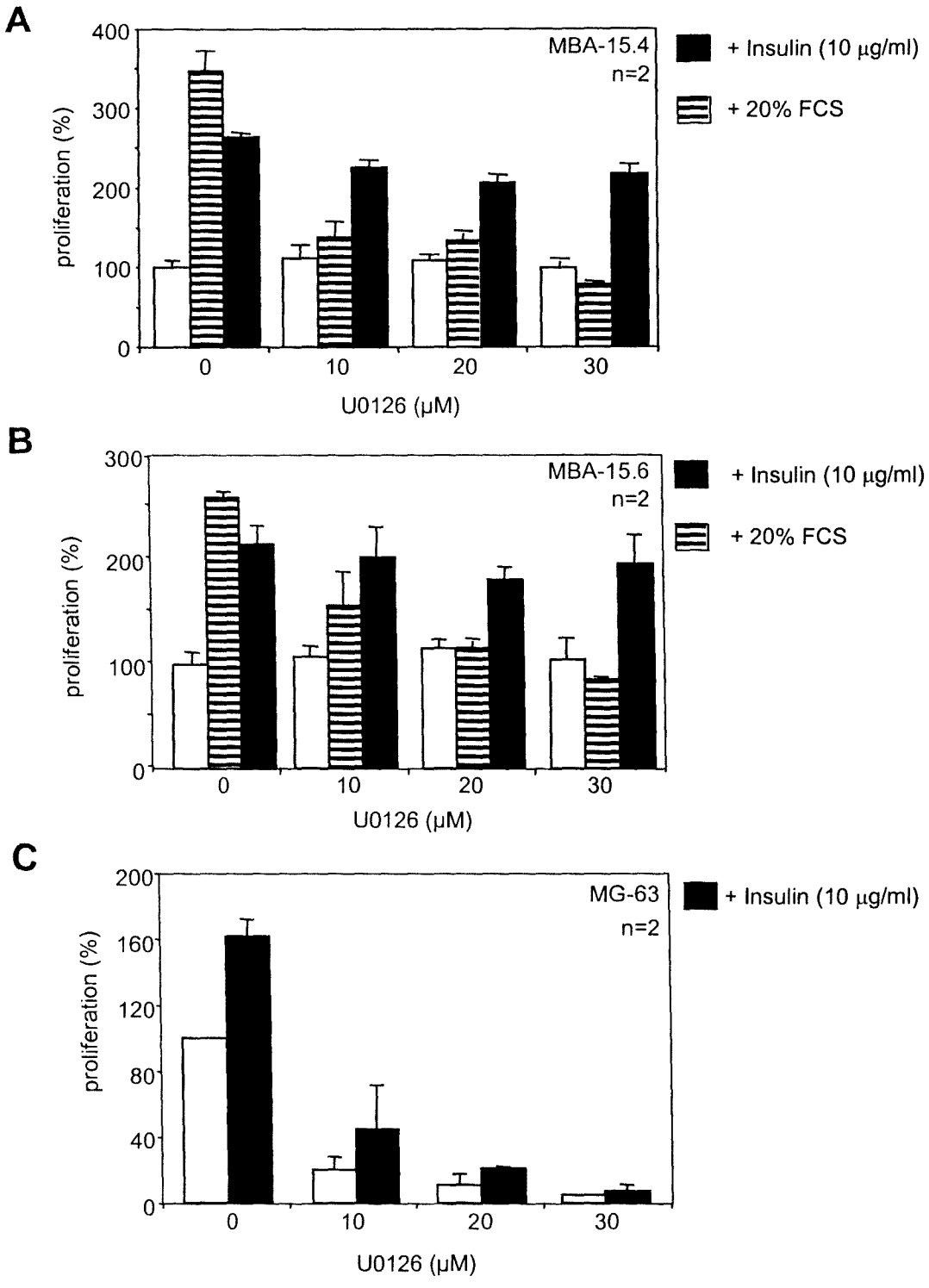

FIG.6. **Comparison of immature vs mature mouse osteoblast and mouse vs human osteoblast responses to blockade of MEK by increasing doses of U0126.** 50% confluent cells were cultured overnight in medium containing 0.5% FCS. MBA-15.4 immature mouse (*A*), MBA-15.6 mature mouse (*B*) and MG-63 immature human (*C*) osteoblast cells were pre-treated for 30 min with increasing concentrations of U0126 (0, 10, 20 or 30 µM) followed by 20% FCS (*A*, *B*) and 10 µg/ml insulin (*A-C*) treatment for a further 24 h. DNA synthesis was measured by [^{3}H] thymidine incorporation. (*A-C*) Results shown are the mean S.D. of two independent experiments, each performed in triplicate and are expressed as percent of respective control.

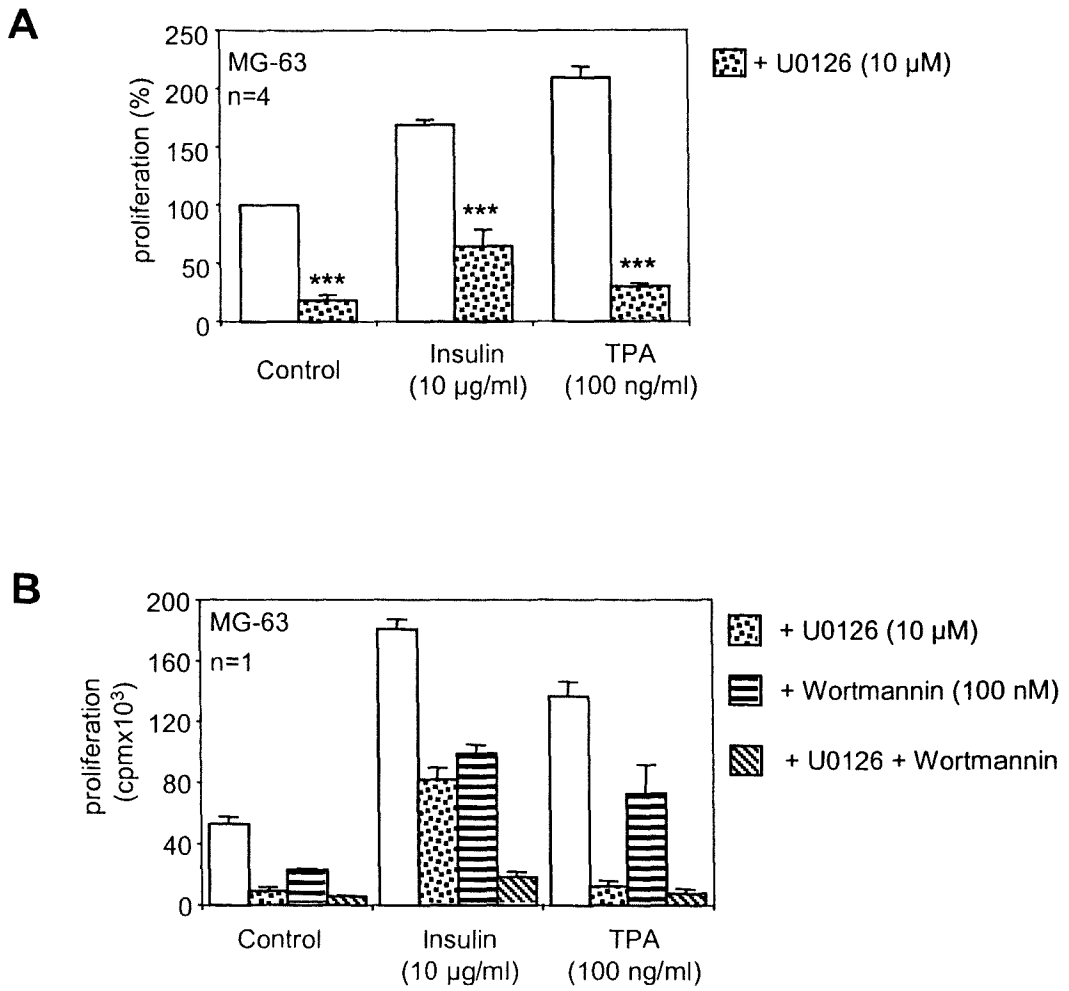

FIG.7. **Insulin mitogenic signaling in human osteoblasts is depended on two separate pathways.** 50% confluent MG-63 cells (*A,B*) were cultured overnight in medium containing 0.5% FCS. Cells were pre-treated for 30 min with or without 10 µM U0126 (*A,B*) or 100 nM wortmannin (*B*) alone, or a combination of the two inhibitors (*B, diagonal hatched bars*). This was followed by a further 24 h treatment without (control) or with 10 µg/ml insulin or 100 ng/ml TPA (*A,B*). DNA synthesis was measured by [^{3}H] thymidine incorporation. (*A*), Results represent the mean ± S.E.M. of four separate experiments, each performed in triplicate and are expressed as percent of respective control. (*B*), Shown is a representative result performed in triplicate. ***P<0.001 (*One-way Anova with Bonferroni post-hoc for multi-group comparison*).

response to insulin (See Fig.16 in discussion). In contrast, TPA-induced proliferation was completely inhibited by U0126 alone and by the combination (*diagonal hatched bars*), but was only partially inhibited by wortmannin alone. This indicates that all proliferative signals, including PI3-kinase signals, converge on ERK and therefore TPA proliferative signaling was completely blocked by MEK inhibition (See Fig.15 in discussion).

8. Dexamethasone inhibits insulin-stimulated cell proliferation

Under most mitogenic conditions, Dex pretreatment inhibits MBA-15.4 and MG-63 osteoblast cell proliferation. We investigated the effect of Dex specifically on insulin-induced proliferation of a mouse preosteoblast cell line, MBA-15.4. Cells were serum starved in 1% FCS for 24 h. Following repeated experiments with this cell line (as published in Hulley et al, 1998) we had established that these cells are increasingly inhibited by 10^{-8} to 10^{-6} M Dex, with 10^{-5} M being toxic and 10^{-6} M the most inhibitory dose. Note that this experiment was done before changing the experimental conditions from serum starving in 1% to serum starving in 0.5% FCS as explained in the results of Fig.1A. Cells were pretreated with 10^{-6} M Dex for 24 h (total Dex treatment was 48 h) before addition of insulin for a further 24 h culture period, followed by [^{3}H] thymidine incorporation. The dose-dependent increase in proliferation in the insulin-stimulated MBA-15.4 cells (Fig.8, *open bars*) are consistent with the result obtained in Fig.1A. Insulin treatment resulted in a 21-45% increase in DNA synthesis. Pretreatment with 10^{-6} M Dex for 24 h (Fig.8, *black bars*) before stimulation with insulin, caused a partial, but not significant inhibition of proliferation at all insulin concentrations. This result indicates that in addition to inhibiting proliferation of exponentially growing cells, Dex pretreatment also inhibited insulin-stimulation of cell proliferation in a preosteoblast cell line, MBA-15.4. However, the decrease in proliferation caused by 48 h Dex treatment was weak and variable, and in subsequent experiments we therefore used 72 h Dex treatments.

9. Effect on osteoblast proliferation of inhibition of protein tyrosine phosphatases (PTPs) by sodium orthovanadate

From our previous work (Hulley et al, 1998) we know that when cells grown in low serum medium are directly stimulated with the mitogenic phorbol ester, TPA (100 ng/ml), 10^{-6} M Dex causes a 35% average decrease in proliferation over 48 h. Concomitant treatment with 1 or 2 doses of sodium orthovanadate over the course of 48 h restored proliferation to between 80-86% of TPA-stimulated levels. A repeat experiment in the MBA-15.4 cells (Fig.9A), indicate a Dex-induced (10^{-6} M) decrease in proliferation of 43%, which was restored to ~80% of TPA-stimulated levels by concomitant sodium orthovanadate (10^{-6} M) treatment over 48 h, confirming the previous result. Sodium orthovanadate is an inhibitor of tyrosine phosphatase activity, indicating that Dex could function by increasing PTP

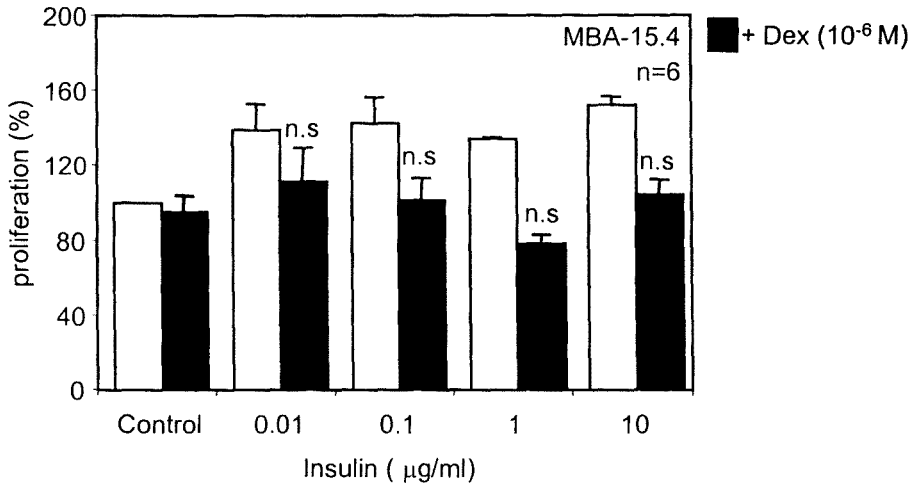

FIG.8. **Dexamethasone inhibits insulin-induced MBA-15.4 preosteoblast cell proliferation.** 50% confluent MBA-15.4 cells were starved overnight in medium containing 1% FCS. Cells were pre-treated with 10^{-6} M Dex (*black bars*) for 24 h where indicated prior to stimulation with different doses of insulin (0.01-10 μg/ml) for a further 24 h. DNA synthesis was measured by [^{3}H] thymidine incorporation. Result shown represents the mean ± S.E.M. of six separate experiments, each performed in triplicate and expressed as percent of respective controls. n.s: not significant when compared to treatment with insulin alone for each respective concentration used (*One-way Anova with Bonferroni post-hoc for multi-group comparison*).

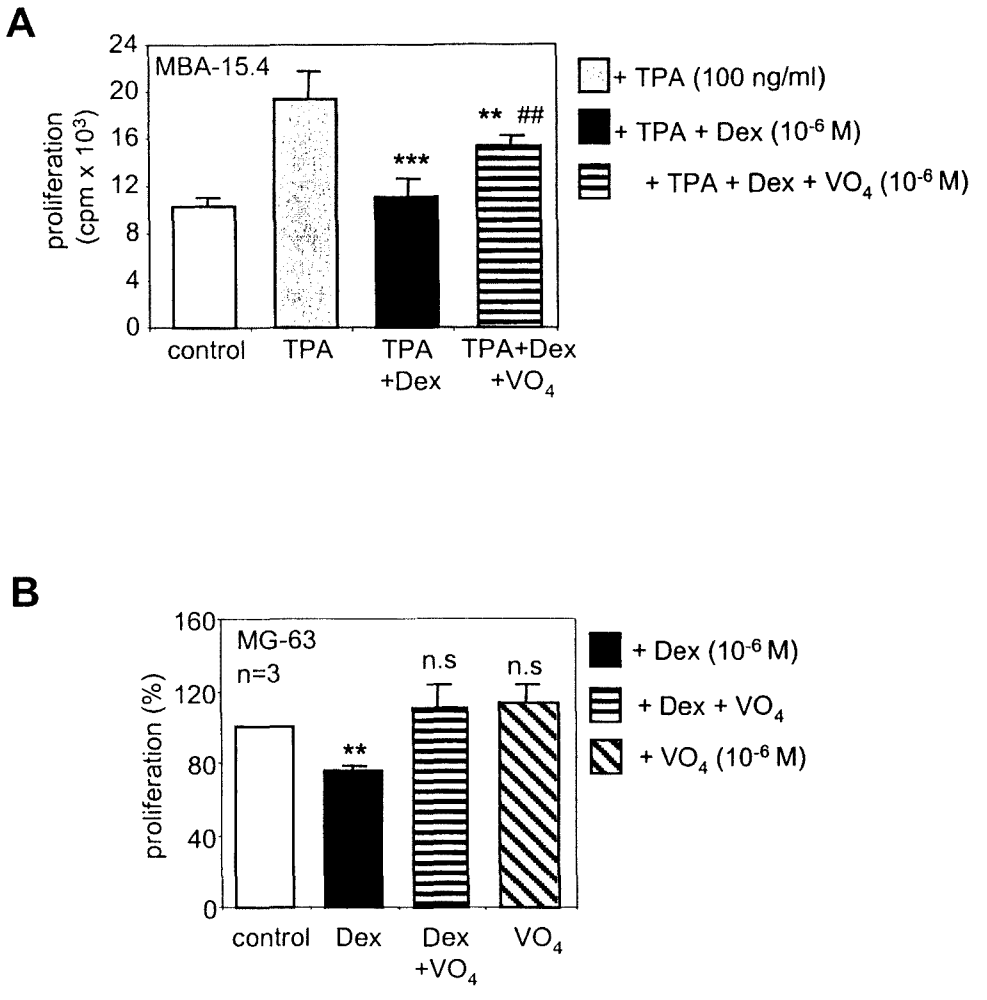

FIG.9. **Sodium orthovanadate reverses inhibition by Dex of TPA- and 10% FCS-induced cell proliferation**. Cells were cultured until 50% confluent followed by overnight serum-starvation in 0.5% FCS. In *A,* the cells were pretreated with Dex (10^{-6} M) alone or Dex combined with 10^{-6} M sodium orthovanadate (*horizontal hatched bars*) for 24 h prior to TPA (100 ng/ml) treatment for 24 h. In *B,* the culture medium was replaced with fresh medium containing 10% FCS and the cells were treated with Dex (10^{-6} M) or sodium orthovanadate (10^{-6} M) alone, or Dex combined with sodium orthovanadate for 24 h. DNA synthesis was measured by [^{3}H] thymidine incorporation. Results shown includes a representative experiment performed in triplicate (*A*) or the mean ±S.E.M. of three (*B*) separate experiments expressed as percent of respective controls. Fig.9B is courtesy of Dr Y Engelbrecht. (*A),* **P<0.01, ***P<0.001 compared with TPA treatment, ## P<0.01 compared with TPA plus Dex treatment. (*One-way Anova with Bonferroni post-hoc for multi-group comparison).* (*B*), **P<0.01 compared with control, n.s. not significantly different to control (*One-way Anova with Dunnett's post-hoc*).

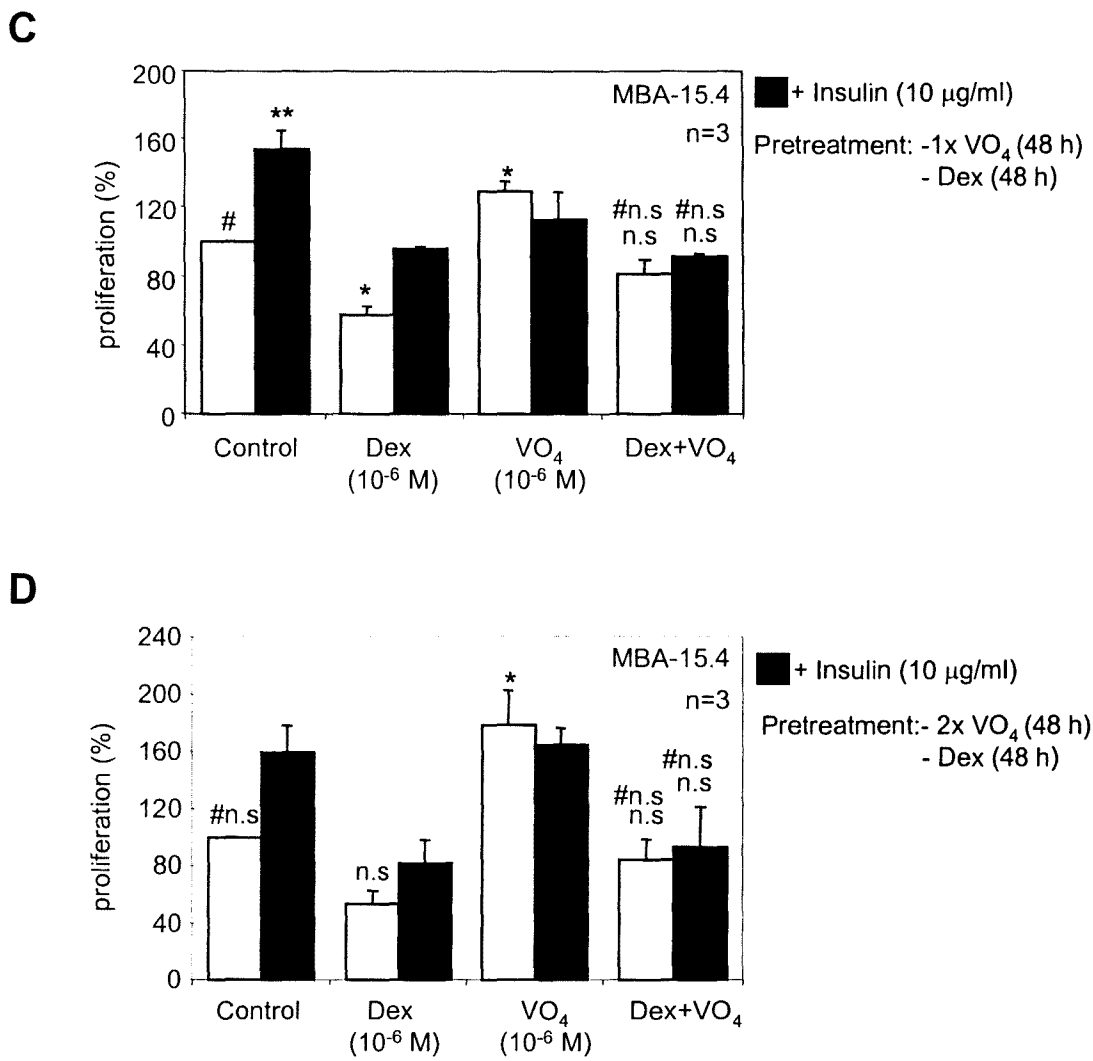

FIG.9 (cont.). **Sodium orthovanadate had no effect on Dex-impaired insulin stimulation of proliferation**. Cells were cultured until 50% confluent followed by overnight serum-starvation in 0.5% FCS. In *C* and *D*, the cells were pretreated with Dex (10^{-6} M) plus one (*C*) or two (*D*) doses of sodium orthovanadate (10^{-6} M) for 48 h prior to insulin (10 µg/ml) treatment for a further 24 h. DNA synthesis was measured by [^{3}H] thymidine incorporation. Results shown are the mean ±S.E.M. of three (*C, D*) separate experiments expressed as percent of respective controls. (*C, D*), #P<0.01, #n.s: not significant, compared with Dex; **P<0.001, *P<0.05, n.s: not significant, compared with insulin plus Dex (*One-way Anova with Bonferroni post-hoc for multi-group comparison*).

levels or PTP activity relative to kinase activity. This was tested further in another preosteoblast cell line, MG-63 human cells. Dex treatment of subconfluent MG-63 cells growing in 10% FCS inhibited proliferation by ~25%. As shown in Fig.9B (*horizontal hatched bars*), concomitant treatment with sodium orthovanadate (10^{-6} M) completely reversed the Dex-induced inhibition, compared to untreated rapidly growing control cells. Therefore proliferation of osteoblasts in response to both TPA and the cocktail of growth factors in FCS was inhibited by 48 h Dex treatment, and inhibition of PTPs effectively reversed this effect. We therefore set out to test the involvement of PTPs in Dex-mediated inhibition of insulin-stimulated proliferation. Culturing serum-starved MBA-15.4 cells in Dex for 48 h had an inhibitory effect, however 48 h Dex pretreatment before 24 h insulin treatment caused a 38% (Fig.9C) and 49% (Fig.9D) decrease in proliferation. Concomitant treatment with 1 (Fig.9C) or 2 doses (Fig.9D) of 10^{-6} M sodium orthovanadate over 72 h failed to restore Dex-inhibited proliferation. Dex treatment for 72 h caused a very severe reduction in osteoblast proliferation. Sodium orthovanadate was unable to reverse this, so PTPs are unlikely to be involved. However, since sodium orthovanadate acts transiently and reversibly, it might be able to reverse a weaker inhibition by Dex. It is apparent in Fig.9C that Dex treatment also inhibited growth of cells under serum-starved conditions, and that insulin partially reversed this. However, this apparent rescue was not significant in Fig.9D and neither vanadate on its own or in combination with insulin was able to reverse this effect of Dex.

10. The PTP, SHP-1 co-immunoprecipitates with ERK, MEK and Raf

Since Dex inhibited FCS- and TPA- stimulated cell proliferation, which was partially corrected by treatment with the PTP inhibitor, sodium orthovanadate, we were interested in the mechanism whereby Dex acts. One possibility could be that Dex may either upregulate PTP levels or PTP activity. To investigate the involvement of possible GC-sensitive PTPs, and their substrates, MBA-15.4 mouse cells were incubated in the presence or absence (control lysates) of 10^{-7} M Dex. Cells were lysed and co-immunoprecipitations were performed as described in the experimental procedures. The PTP, SHP-1 has been implicated as a negative regulator of the insulin receptor (Bousquet et al, 1998) and can be upregulated by GCs (Cambillau et al, 1995). SHP-1 co-immunoprecipitated with ERK (Fig.10B), MEK (Fig.10D) and Raf (Fig.10F), suggesting that SHP-1 may be an important regulator of osteoblast proliferation. SHP-1 requires association with tyrosine phosphorylated proteins for activation. MEK is not tyrosine phosphorylated but rather serine phosphorylated, suggesting that SHP-1 association with substrates in the mitogenic cascade is due to the formation of a complex.

11. The PTP, PTP-1B co-immunoprecipitates with ERK and MEK but not Raf

The literature provides considerable support for a physiological role for PTP-1B in the negative regulation of the insulin action pathway (Goldstein et al, 2000). To further investigate the involvement

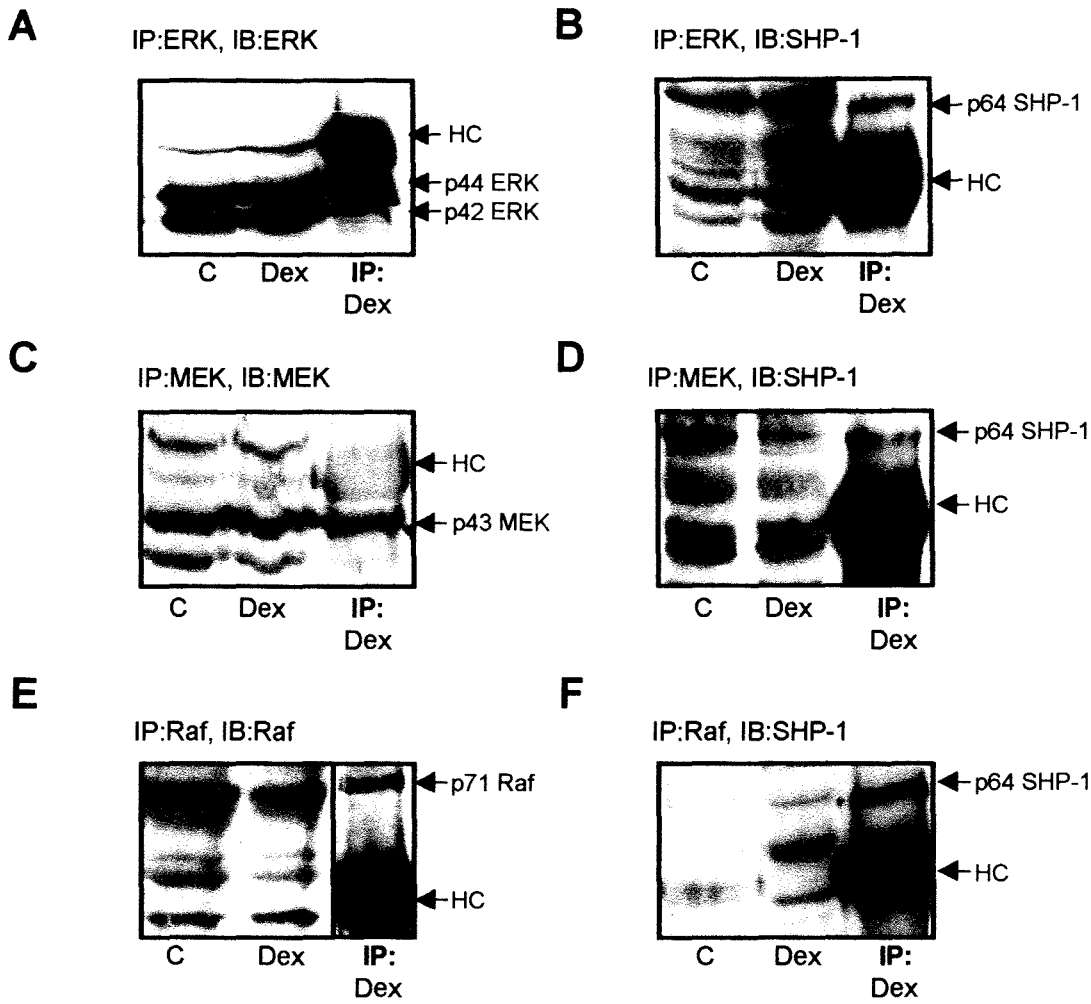

Fig.10. **Co-immunoprecipitation of SHP-1 with ERK, MEK and Raf-1 in MBA-15.4 preosteoblast cells following Dex treatment.** The cells were grown with 10% FCS until 50% confluent, and then cultured for a further 48 h in fresh 10% FCS medium (control lysate lane 1), some containing 10^{-7} M Dex (lysate lane 2 and 3). Cells were lysed and subjected to immunoprecipitation using anti-ERK (*A,B*), anti-MEK (*C,D*) and anti-Raf (*E,F*) antibodies, coupled to protein A agarose beads. Beads were washed, boiled in sample buffer to release protein and proteins were analysed by Western Blotting using antibodies for ERK, MEK, Raf and SHP-1 (as indicated) and detected by ECL enhancement and chemiluminescence. Lysate lanes in (F) were overexposed to detect relatively low levels of Raf-1 (IP, immunoprecipitation; IB, immunoblotting; HC, IgG heavy chain, Mr 50kDa).

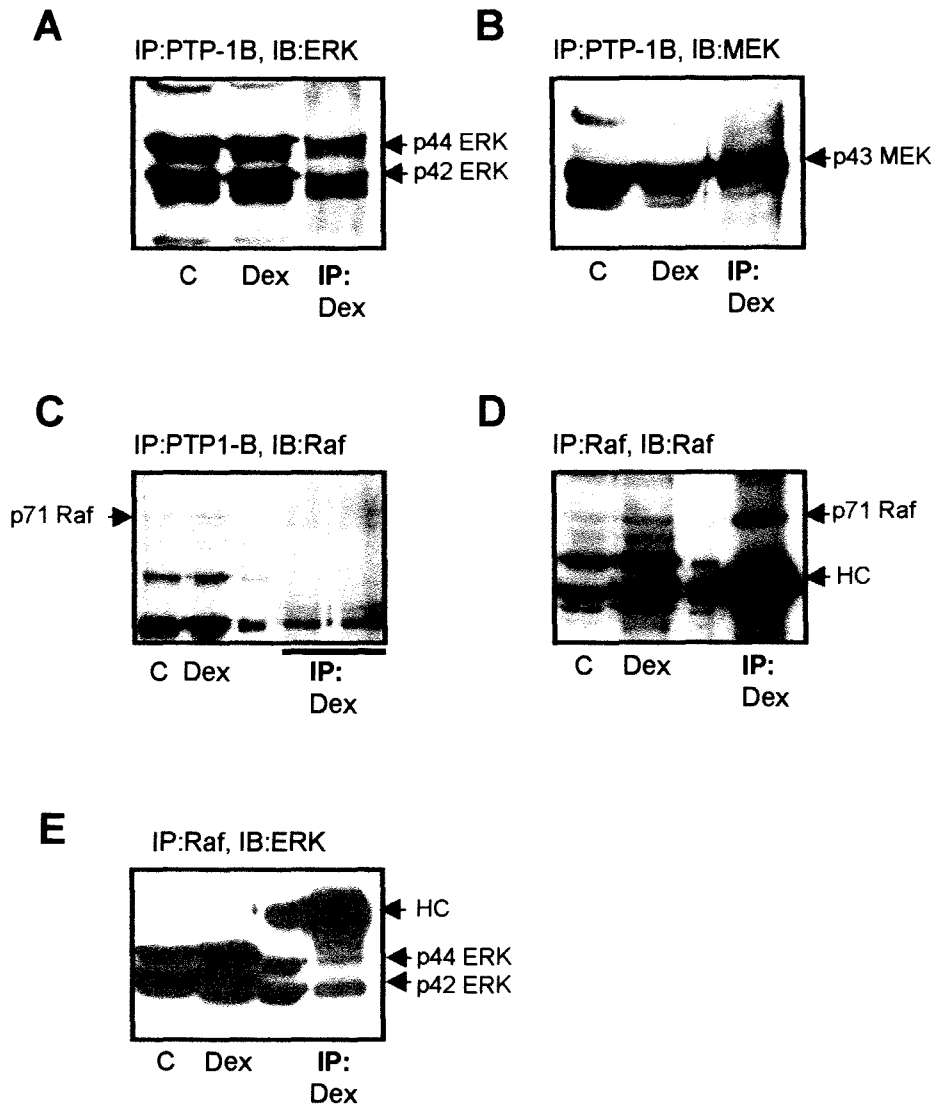

Fig.11. **Co-immunoprecipitation of ERK and MEK with PTP-1B in MBA-15.4 preosteoblasts following Dex treatment.** The cells were grown with 10% FCS until 50% confluent, and then cultured for a further 48 h in fresh 10% FCS medium (control lysate lane 1), some containing 10^{-7} M Dex (lysate lane 2 and IP). Cells were lysed and subjected to immunoprecipitation with anti-PTP-1B (*A, B, C*) and anti-Raf (*D, E*) antibodies. Immunoblots were probed with antibodies to ERK, MEK and Raf (as indicated). Lane 3 is an open lane in C,D and E. (IP, immunoprecipitation; IB, immunoblotting; HC, IgG heavy chain, Mr 50kDa).

of possible vanadate-sensitive PTPs in osteoblast mitogenic signaling, we repeated the above experiment, looking at PTP-1B as a candidate. ERK (Fig.11A) and MEK (Fig.11B), but not Raf (Fig.11C), co-immunoprecipitated with PTP-1B. Once again, MEK cannot serve as a direct substrate for PTP-1B, therefore this indicates multiprotein complex formation. To confirm that the 71kDa protein seen in Fig.10E corresponded to Raf-1 and also to check sensitivity of the technique, cells were treated with 10^{-7}M Dex, immunoprecipitated with anti-Raf antibody and immunoblotted with anti-Raf (Fig.11D) and anti-ERK (Fig.11E) antibody. Although we failed to show that PTP-1B associates with Raf-1, this result demonstrated that Raf-1 is present (Fig.11D) and that our antibody is active. ERK cannot serve as a substrate for Raf-1, and yet the two kinases co-immunoprecipitated. This gave further confirmation that the kinases immunoprecipitated as a complex.

12. SHP-1 associates with active, tyrosine phosphorylated ERK

In Fig.10B, we have shown that SHP-1 co-immunoprecipitates with ERK, thus indicating that this phosphatase may play an important role in the down-regulating of preosteoblast proliferation. Using an antibody that detects active, tyrosine phosphorylated ERK-1/2 we found that ERK does co-immunoprecipitate with SHP-1 under the mitogenic conditions used (5 min, 20% FCS treatment, Fig.12A and B). This indicates that SHP-1 associates with active ERK and may dephosphorylate it, suggesting that it could play a role in the inhibition of preosteoblast proliferation. In Fig.12.C and D, we immunoprecipitated with an antibody that detects all tyrosine phosphorylated proteins. In the presence of 20% FCS, p42 and p44 ERK is strongly phosphorylated on both tyrosine and threonine residues (Fig.12D, lane 2). Fig.12D indicates that the anti-active ERK antibody specifically detected ERK tyrosine residues. Although very low levels of protein were detected in the immunoprecipitation compared to treated control lysates, active ERK was present, confirming that SHP-1 associates with active, tyrosine phosphorylated ERK (Fig.12D).

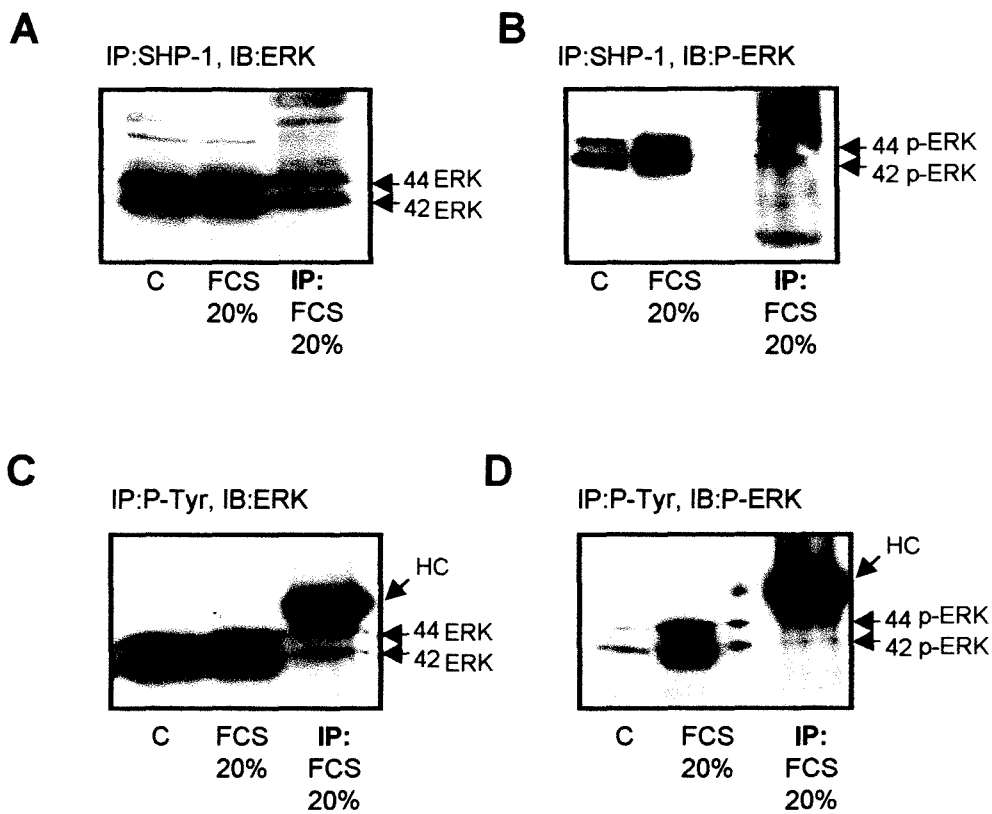

Fig.12. **Association of SHP-1 with active, tyrosine phosphorylated ERK-1/2.** MBA-15.4 cells were grown in 10% FCS until 50% confluent, then serum starved in 1% FCS for 24 h, and were left untreated (control lysate lane 1) or stimulated with 20% FCS for 5 min (lysate lane 2 and IP). Cell lysates were subjected to immunoprecipitation by either anti-SHP-1 (*A, B*) or anti-phosphotyrosine antibodies (*C, D*) and proteins detected, following Western blotting, with either anti-active ERK (*B, D*) or ERK (*A, C*) antibodies. (IP, immunoprecipitation; IB, immunoblotting; HC, IgG heavy chain, Mr 50kDa).

CHAPTER 4

DISCUSSION

In mammals insulin functions as a mitogen for many cells e.g. epidermal cells, developing neurons and symphathetic neuroblasts, preadipocytes and capillary endothelial cells (Lev-Ran, 1998). Insulin also seems to be an important osteoblast mitogen. Insulin deficiency in Type I diabetes in humans (Mathiassen et al, 1990; Hough et al, 1987) and rats (Hough et al, 1981; Reddy et al, 2001) causes severe osteopenia. In contrast, there is still a lot of controversy about bone mass changes in insulin resistant or type II diabetes patients. Bone mineral density in these individuals could be increased, decreased (Buysschaert et al, 1992) or unchanged (Sosa et al, 1996) compared to sex-and age-matched subjects (Piepkorn et al, 1997). However, several findings indicate that overweight type II diabetics have a normal or increased bone mineral density (Jensen et al, 2001). Protection against osteopenia might be due to increased production of estrogen in the subcutaneous fat tissue, the stress of weight bearing on the skeleton or the high circulating levels of insulin serving as an osteoanabolic agent (Barret-Connor and Kritz-Silverstein, 1996). The insulin mimetic, vanadium, has been shown to protect against the bone damaging effects of steroid treatment *in vitro* (Hulley et al, 1998) and *in vivo* (Hulley et al, 2001). A critical role for insulin signaling in osteoblasts has recently been demonstrated in the IRS-1 knockout mouse. IRS-1 deficient mice showed impaired proliferation and differentiation resulting in low-turnover osteopenia (Ogata et al, 2000). Osteoblastic IRS-1 is thus important for maintaining bone turnover, further confirming the importance of insulin as a bone mitogen. However, the mitogenic signaling pathways used by insulin in osteoblasts remain poorly described. Therefore, the aim of this study was to investigate the effect of insulin on proliferation, to map out the potential signaling targets of insulin, to look at the effects of glucocorticoids on insulin-induced osteoblast proliferation and then to examine whether the insulin mimetic, vanadate, would effectively combat GC damage.

Selection of experimental system

To determine whether insulin can stimulate osteoblast proliferation, [^{3}H] thymidine incorporation was measured in different osteoblast cell lines. This was done in both relatively immature (MBA-15.4 mouse and MG-63 human), more mature (MBA-15.6 mouse) and differentiated (UMR-106 rat) osteoblast cell lines. These are all well characterized osteoblast cell lines, expressing different levels of osteoblast differentiation markers. The mouse marrow derived MBA-15.4 cells produces low levels of alkaline phosphatase and PTH receptors compared to MBA-15.6 osteoblasts expressing high alkaline

phosphatase activity and levels of PTH receptors (Fried et al, 1993). The human MG-63 osteosarcoma preosteoblasts express TGF-β- and 1,25-dihydroxyvitamin D3- inducible alkaline phosphatase activity (Clover and Gowen, 1994; Bonewald et al, 1992). The rat osteoblast-like osteosarcoma cell line UMR-106 express high levels of receptors for vitamin D, GH, leukemia inhibitory factor and PTH (Martin et al, 1979). Insulin stimulated a marked increase in proliferation in the less differentiated MBA-15.4, -15.6 and MG-63 cell lines. Although a wide range of insulin concentration was used, the mature UMR-106 cells was not very insulin responsive and showed a weak increase in proliferation. Possible explanations are that the proliferative potential of these bone cells in response to insulin correlates with their stage of differentiation, with the immature cells being more insulin responsive than the more differentiated cells. However, these are spontaneously transformed cell lines with defects in their proliferative control pathways. They never stop growing, unlike normal osteoblasts, and all results must be interpreted with this in mind. Differences observed in response to insulin represent differences in transformation processes between cell lines, but since all three immature cell lines were more responsive to insulin than UMR-106 cells, it is possible that insulin responsive pathways are up-regulated in younger cells. This could be due to increased numbers of IR or IGF receptors, or elevated levels of IRS-1. The insulin concentrations used were between 0.01-10 μg/ml (1.74 nM-1.74 μM). Although, commonly used in the literature at 100 nM, we were not sure how much of the insulin reached the cells and therefore used a wide range of concentrations. Normal fasting plasma insulin levels in rats (Hough, 1981) and humans (Greenspan and Strewler, 1997) averages 0.4 ng/ml (69pmol/L), and can go up to 4 ng/ml (690pmol/L) after standard meals. The doses that we used were therefore relatively high compared to normal plasma insulin levels.

Insulin-induced proliferation of the MBA-15.4 immature mouse osteoblast cell line

The MBA-15.4 mouse preosteoblastic cell line was strongly insulin sensitive. The phorbol ester TPA and 20% FCS were included as positive controls. Both of these mitogens markedly increased proliferation above control levels. However, insulin increased proliferation was comparable to TPA-induced DNA synthesis. We also established that although the increase in proliferation in response to different insulin concentrations in 1% serum conditions was comparable to the increase seen when serum starving in 0.5% FCS, the lower serum conditions produced more consistent results.

Insulin can activate proliferation through both the Raf-MEK-ERK cascade, or through the initial component of the metabolic pathway comprising IRS-1 and PI3-kinase. Therefore the next question was whether the same signaling pathways are involved in insulin-induced proliferation in osteoblasts. To determine this, two specific inhibitors, U0126, a MEK-1 and -2 inhibitor, and wortmannin, a PI3-kinase inhibitor, were used to establish the pathway by which insulin signals to proliferation. Increased

proliferation in response to 20% FCS was completely MEK-dependent and PI3-kinase-independent. A completely unexpected finding was made when insulin signaling to proliferation was completely unaffected by inhibition of both MEK and PI3-kinase activation in MBA-15.4 preosteoblasts. This was repeated and confirmed 18 times in triplicate. Interestingly, although a resistance to MEK-inhibition was always observed, in 6 independent experiments an increase in proliferation was also seen when cells were treated with a combination of insulin and the MEK inhibitor, U0126. This was investigated further by using the two inhibitors in combination, thus inhibiting both MEK and PI3-kinase at the same time. With 20% FCS, using a combination of the two inhibitors was the same as inhibiting MEK alone which confirmed that FCS-induced stimulation of DNA synthesis was routed through the MEK-ERK pathway (Fig.13). However, the two inhibitors alone or in combination failed to have an effect on insulin-induced cell proliferation. These observations indicate that insulin-induced DNA synthesis in MBA-15.4 mouse preosteoblasts does not involve MEK-ERK or PI3-kinase activation, and suggest the involvement of an alternative pathway.

The resistance of insulin-induced proliferation to inhibition and also the increased proliferation that we frequently observed when blocking MEK may indicate that there is a negative feedback signal at the level of or downstream of MEK (Fig.14). When MEK is inhibited, this feedback signal is removed, allowing increased proliferation via an alternative pathway. Reports indicate that insulin activation of ERK causes hyperphosphorylation of Sos, promoting dissociation of constitutively bound Grb2-Sos complexes (Goalstone and Draznin, 1998). Dissociation of Grb2-Sos results in the desensitization of Ras to prolonged activation of growth factor stimulation, whereas MEK inhibition blocks this event, resulting in prolonged Ras activation (Langlois et al, 1995). This may indicate that in MBA-15.4s, insulin signals through a non-MEK, non-PI3-K Ras-dependent pathway.

In the literature U0126 is commonly used at 10 μM. Just to verify that the concentration of U0126 was optimal, we used two to three times higher concentrations. In the MBA-15.4, FCS-induced osteoblast proliferation was potently inhibited by 10 μM U0126 and showed a further dose-dependent sensitivity to MEK-inhibition. Insulin-induced DNA synthesis was unaffected by an increase in the concentration of the inhibitor. From this result we concluded that the time of treatment and the dose of U0126 was optimal, supporting our observation of the involvement of a MEK-independent pathway in insulin activated cell proliferation.

Role of ERK in the MBA-15.4 osteoblast response to insulin

Insulin markedly increased proliferation in MBA-15.4 osteoblasts in a MEK-and PI3-kinase-independent manner. We therefore wondered whether insulin could activate ERK in bone cells. In

many cell types previously studied, insulin treatment induces the activation of ERK (Lazar et al, 1995). Likewise, in osteoblasts, insulin stimulated ERK-1/2 activity, but this activation was weak and transient. Compared to insulin, TPA and FCS strongly stimulated ERK activity, and ERK phosphorylation was still detectable 24 h after treatment. This suggested that in comparison to known mitogens, ERK activation may not be so important in insulin-induced proliferation of MBA-15.4 osteoblasts.

Wortmannin, the PI3-kinase inhibitor, had no effect on either insulin or 20% FCS-induced DNA synthesis. Therefore, to exclude the possibility that the inhibitor was not working optimally, activation of Akt/PKB, a well-established downstream target of PI3-kinase, was investigated (Burgering and Coffer, 1995). Insulin treatment caused a prolonged increase in Akt/PKB phosphorylation. Pretreatment with the PI3-kinase inhibitor completely blocked insulin signaling to Akt/PKB. This indicates that insulin mediates the activation of a wortmannin-sensitive PI3-kinase upstream of Akt/PKB, which is not involved in proliferation of MBA-15.4 osteoblasts.

Insulin can signal through two major pathways, both of which may involve ERK as a downstream substrate. Next, we used the inhibitors to further investigate the role that ERK plays in bone cells. Using U0126, the MEK-1 and -2 inhibitor, we found immediate inhibition of insulin- and FCS-stimulated ERK activity, and this inhibition lasted for 24 h, in agreement with a negative effect on cell cycle. Also, the PI3-kinase inhibitor, wortmannin, decreased insulin-induced ERK activity at all time points but only visibly affected 20% FCS-induced ERK activation after 24 h. In the MBA-15.4 preosteoblast cell line, both insulin and FCS activation of ERK was MEK-dependent. Further, whereas insulin stimulation of ERK was also PI3-kinase-dependent, FCS stimulation seemed to only slightly involve PI3-kinase, since wortmannin only visibly inhibited ERK after 24 h. Therefore insulin signaling to ERK was dependent on both MEK and PI3-kinase, but this had no effect on cell proliferation. In contrast, both ERK activation and proliferation induced by 20% FCS was almost entirely dependent on MEK-ERK (Fig.13).

Comparison with a more mature cell line, MBA-15.6

In the immature MBA-15.4 mouse cell line, the MEK-ERK and PI3-kinase signaling pathways appeared not to be essential for insulin's effect on DNA synthesis. We repeated the experiment with increasing U0126 concentrations in the more mature mouse cell line, MBA-15.6. Again 20% FCS-induced proliferation was abolished by the inhibitor, whereas insulin was resistant to all concentrations of the inhibitor. It therefore seems that in both an immature and more mature mouse cell line the classical signaling pathways are not involved in insulin- stimulated proliferation.

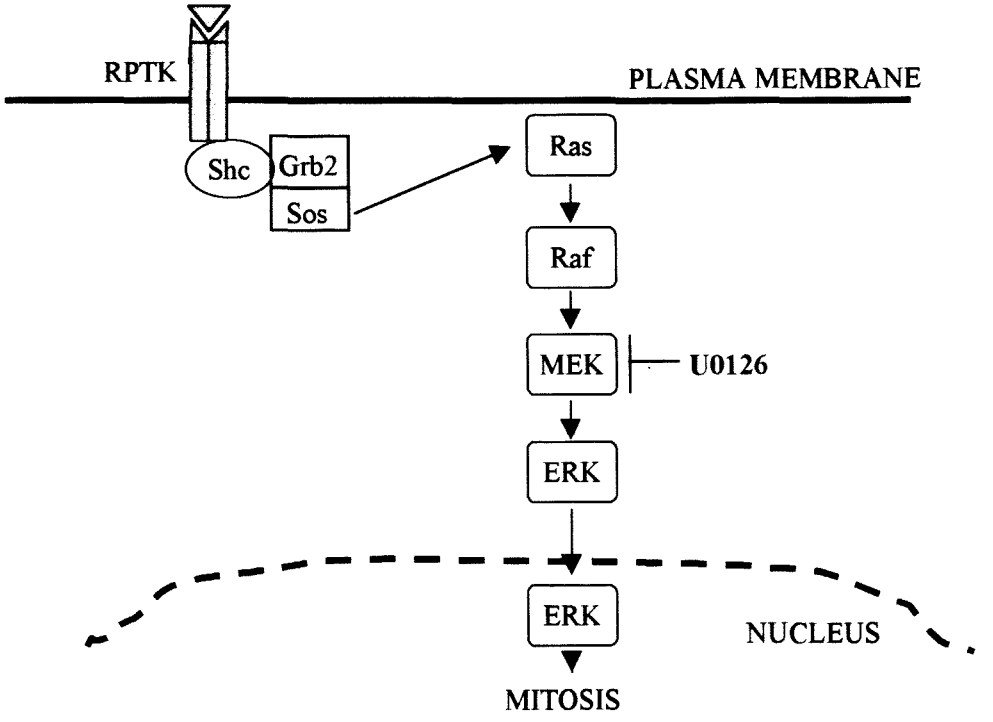

Figure 13: The signal transduction cascade stimulated by growth factors such as 20% FCS in MBA-15.4 osteoblasts. The MEK inhibitor, U0126 blocked proliferation.

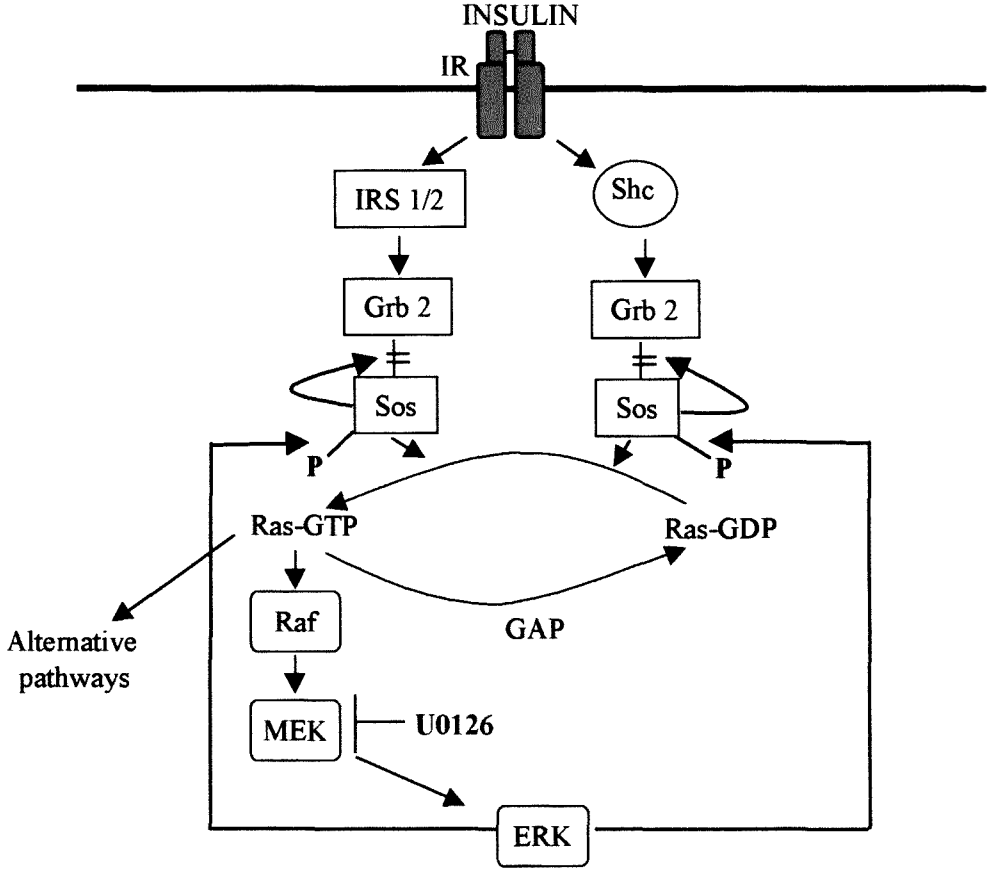

Figure 14: The classical insulin signal transduction pathways (*in black*). Active ERK can hyperphosphorylate Sos, causing dissociation of Grb2-Sos complexes (*in red*). The negative feedback terminates the insulin signal and deactivates Ras. MEK inhibition blocks the negative signal from ERK, resulting in a prolonged Ras activation (Modified from Goalstone and Draznin, 1998).

Comparison with a human immature osteoblast cell line, MG-63

The MG-63-human osteoblast cell line, like the MBA-15.4, is considered to be a less differentiated or immature cell line. Both insulin and TPA, which was included for comparison, stimulated proliferation. However, in contrast to the mouse cell line, the human cells responded to MEK inhibition with a marked decrease in proliferation. While this finding may be simply due to differences between immortal cell lines or a drug effect of U0126, it also may indicate species differences, with the mouse cells being independent and the human cells being dependent on MEK for DNA synthesis. Wortmannin, the PI3-kinase inhibitor, partially blocked both TPA- and insulin- induced proliferation. However, MEK inhibition completely blocked TPA and partially prevented insulin stimulated cell proliferation. With TPA treatment, blocking both pathways at the same time decreased proliferation to the same extent as MEK inhibition alone. Phorbol esters like TPA can mediate their effects through the direct activation of PKC-Raf-MEK-ERK, bypassing receptor signaling (Fig.15). However, TPA stimulates PI3-kinase in a number of cell types, including 3T3-L1 adipocytes (Nave et al, 1996) and JB6 cells (Huang et al, 1998). This is probably due to transactivation of receptors by TPA. Recently, it has been shown that TPA cannot activate ERK or cell cycle in the absence of EGF receptor (Chen et al, 2001). In MG-63 osteoblasts, it seems that although both MEK and PI3-kinase are involved in TPA signaling to proliferation, the MEK-ERK limb of the pathway is dominant. Therefore although some of the signal goes through PI3-kinase, all of the signal eventually goes through ERK (Fig.15). This cross-talk of PI3-kinase with Ras-Raf-MEK has been reported for many cell lines, including 3T3-L1 adipocytes (Suga et al, 1997), L6 rat skeletal muscle (Cross et al, 1994) and COS cells (Wennstrom and Downward, 1999). In contrast, whereas the two inhibitors on their own both partially inhibited insulin-stimulated proliferation, combining the two completely blocked DNA synthesis, as reported for the human neuroblastoma cell line, SH-SY5Y (Kurihara et al, 2000). This indicated that in the human cell line, insulin signals via two separate pathways, one going through MEK-ERK and one dependent on PI3-kinase and inhibition of both totally accounts for the increase in proliferation (Fig.16).

Looking at Fig 6C and Fig 7A and B, there is evidence in all three experiments of a possible drug effect of U0126, because of the severe inhibition of basal (0.5% FCS) DNA synthesis. U0126 had a much greater inhibitory effect on TPA induced-proliferation than on insulin-induced proliferation, which could be explained by a partial decrease by drug effect, seen in 0.5% FCS controls, insulin and TPA- treated samples, but with an additional true inhibition of MEK-ERK in the TPA samples. However, the relative decrease in the basal DNA synthesis under conditions of 0.5% FCS culture is ~85%, statistically no different from the ~85% relative decrease in proliferation seen with TPA (one-way ANOVA with Bonferroni Posthoc, results not shown). The relative decrease produced by U0126 on insulin signalling was only ~50%, which was statistically less than the change produced in the other

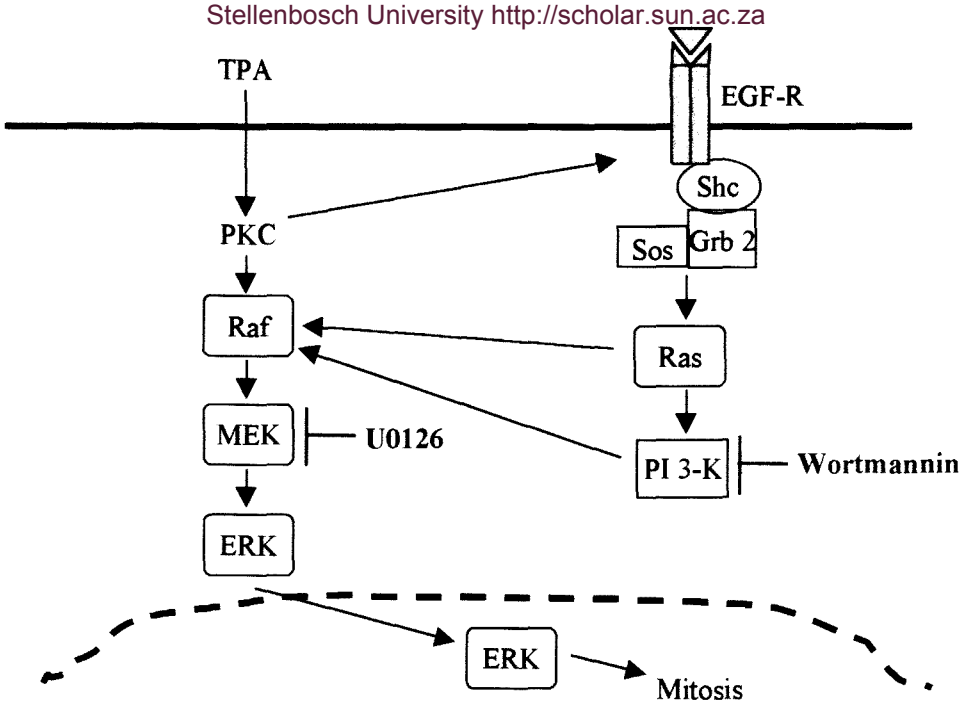

Figure 15: The signal transduction pathways stimulated by the phorbol ester, TPA, in a human osteoblast cell line, MG-63. TPA activates PKC independently of receptor activation. A recent report indicates that TPA activation of ERK requires EGF receptor activation, suggesting possible transactivation of the receptor via TPA. This can lead to PI3-K activation. Blocking PI3-kinase partially prevented DNA synthesis, whereas MEK inhibition completely blocked proliferation. This indicates that some of the TPA signal goes though PI3-K, which feeds onto MEK-ERK.

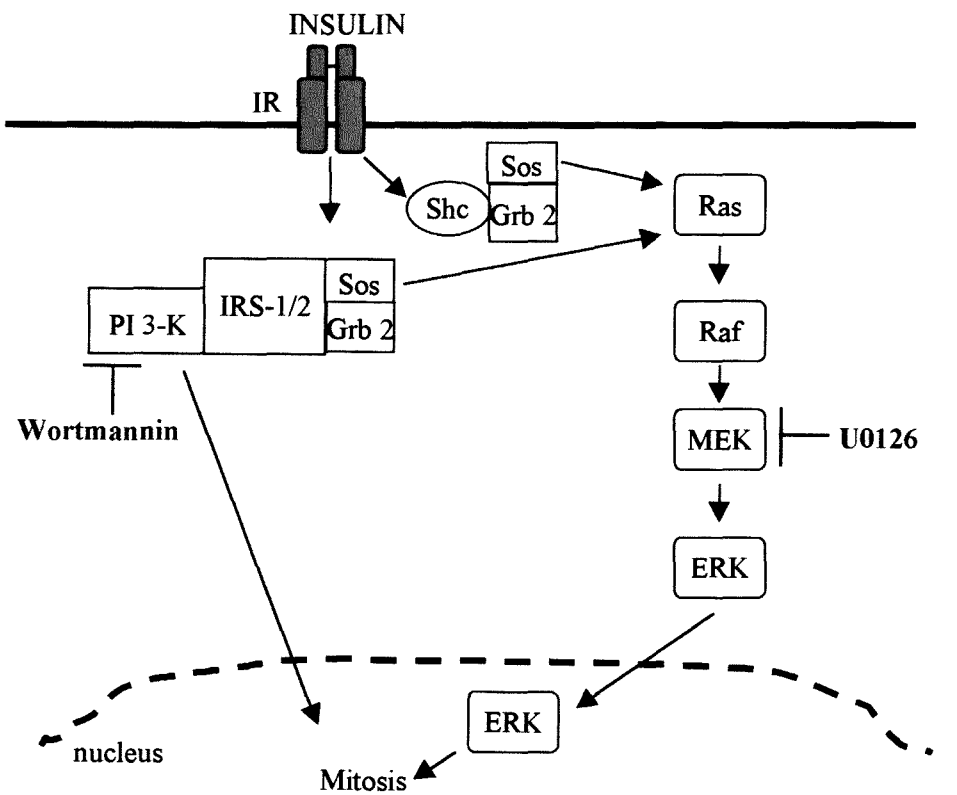

Figure 16: The signal transduction pathway stimulated by insulin in a human osteoblast cell line, MG-63. Insulin signals through PI3-kinase and MEK-ERK and inhibition of both pathways completely abolished proliferation.

2 treatment groups. A true drug effect should have affected all 3 to the same extent, with additional inhibitory effects seen on TPA only. We therefore interpret the data as indicating a very strong dependence on MEK of basal proliferation and that induced by TPA, whereas insulin signalling is only partially MEK dependent. This needs to be further investigated to conclusively rule drug effect out or in.

Effects of GCs and PTP inhibition on insulin-induced proliferation in immature mouse osteoblasts, MBA-15.4

Clinically, long-term high dose steroid use is the most common cause of secondary osteoporosis. Our group have shown that *in vitro* GCs impair osteoblast proliferation of an immature cell line, MBA-15.4, which correlated with a decreased response to growth factors and a blunted ERK-1/2 activation (Hulley et al, 1998). Sodium orthovanadate repaired GC-impaired ERK activation and cell proliferation by inhibiting protein tyrosine phosphatases thus prolonging the activation of the kinase cascade. This was confirmed in a rat model of steroid-induced osteoporosis, where daily vanadate supplementation improved bone strength and bone formation, thus providing protection against the negative effects of GC treatment (Hulley et al, 2001). Vanadate may act by blocking GC-induced upregulation of PTPs or preventing inhibition of tyrosine phosphorylated substrates, desensitizing the pathway to mitogenic stimulation. Moreover, the ability of vanadium to act as a insulin mimetic makes it useful as treatment of diabetes in humans (Cohen et al, 1995; Goldfine et al, 1995) and animals (Meyerovitch et al, 1987; Brichard et al, 1988). Inhibition of PTPs and increased insulin receptor phosphorylation are postulated to be the mechanism of action whereby vanadium exert its insulin-enhancing effect. Candidate phosphatases that negatively regulate this pathway include PTP-1B (Goldstein et al, 2000) and SHP-1 (Bousquet et al, 1998). PTP-1B knockout mice are resistant to obesity when fed a high-fat diet and showed increased insulin sensitivity (Elchebly et al, 1999). Overexpression of PTP-1B in adipocytes suppress PI 3-kinase activation (Venable et al, 2000). This dampening of important regulatory signals stresses the role that phosphatases can play in human disease.

Candidate phosphatases that have been reported to negatively affect growth factor and insulin signaling pathways, include SHP-1 and PTP-1B. With co-immunoprecipitation, we were able to demonstrate association between molecules in the Raf-MEK-ERK cascade and tyrosine phosphatases. We successfully demonstrated that ERK and MEK co-immunoprecipitate with PTP-1B. However, although we were unable to demonstrate this for Raf, Raf was detectable in these cells. Co-immunoprecipitation of MEK with PTP-1B, which is serine and not tyrosine phosphorylated, and ERK with Raf, which is not directly upstream of ERK, indicated that a multiprotein complex is pulled down. Likewise, SHP-1 co-immunoprecipitated with ERK, MEK and also Raf respectively. We could

therefore say that PTP-1B and SHP-1 are present in our cells and associate with molecules in the mitogenic kinase cascade, and therefore may be vanadate targets. We further confirmed that SHP-1 is associated with active, tyrosine-phosphorylated ERK. This may indicate that SHP-1 associates with active tyrosine-phosphorylated ERK in order to dephosphorylate it. However, more direct evidence of this is needed before such a conclusion can be made.

The next question that I wanted to answer is whether GC treatment inhibits insulin-induced osteoblast proliferation in the same way as it inhibits growth factor induced proliferation. Pretreatment with different concentrations of the GC dexamethasone dose-dependently decreased insulin-induced proliferation in the MBA-15.4 preosteoblasts. Likewise, in the human MG-63 preosteoblasts, Dex inhibited proliferation of cells growing in 10% FCS. The PTP inhibitor, sodium orthovanadate completely reversed GC-impaired proliferation of the MG-63s. Also, we know that vanadate corrects the decrease in TPA-induced proliferation caused by GC treatment (Hulley et al, 1998). We were able to demonstrate in both a mouse and human immature osteoblast cell line that vanadate can oppose the negative effects that steroids have on TPA and 10% FCS treatment.

Since Dex inhibited insulin-induced proliferation, we were interested to see whether vanadate would protect against steroid-impaired proliferation. It is not possible to maintain live cultures in the complete absence of FCS and since FCS is a cocktail of GFs, we lowered the serum concentration to prevent our insulin responses from being masked by high GF levels. We initially serum starved in 1% FCS before changing the experimental conditions to serum starving in 0.5% FCS, because this gave a more reproducible insulin stimulation. Prolonging the time of exposure to Dex from 48 h to 72 h, and treating concurrently with insulin and vanadate for the last 24 h, resulted in a marked decrease in proliferation, but with no visible salvage by sodium orthovandate. Next we prolonged the vanadate treatment from once for 24 h to twice over a period of 48 h. However the vanadate was still unable to correct the steroid-impaired proliferation, and since vanadate functions by inhibition of PTPs this indicated that vanadate-sensitive phosphatases may not be involved. We therefore concluded that TPA- and growth factor- induced ERK activation and cell proliferation is markedly impaired by GCs, while co-treatment with vanadate provides protection by reversing the effects of GCs. However this is not true for insulin signaling in osteoblasts which were growth-impaired by steroids, but fairly resistant to the effects of vanadate. GCs decrease the response to growth factors such as TPA and 10% FCS of the mitogenic signaling cascade, causing a blunted Raf-MEK-ERK activation and this was repaired by co-treatment with the PTP inhibitor, vanadate (Hulley et al, 1998). In contrast, vanadate had no effect on GC-impaired insulin signaling. Although we showed that the PTPs, PTP-1B and SHP-1, associates with Raf, MEK and ERK, this is unlikely to be important for insulin signaling, since insulin does not use the Raf-MEK-ERK pathway for proliferation in MBA-15.4 and 15.6 mouse osteoblasts.

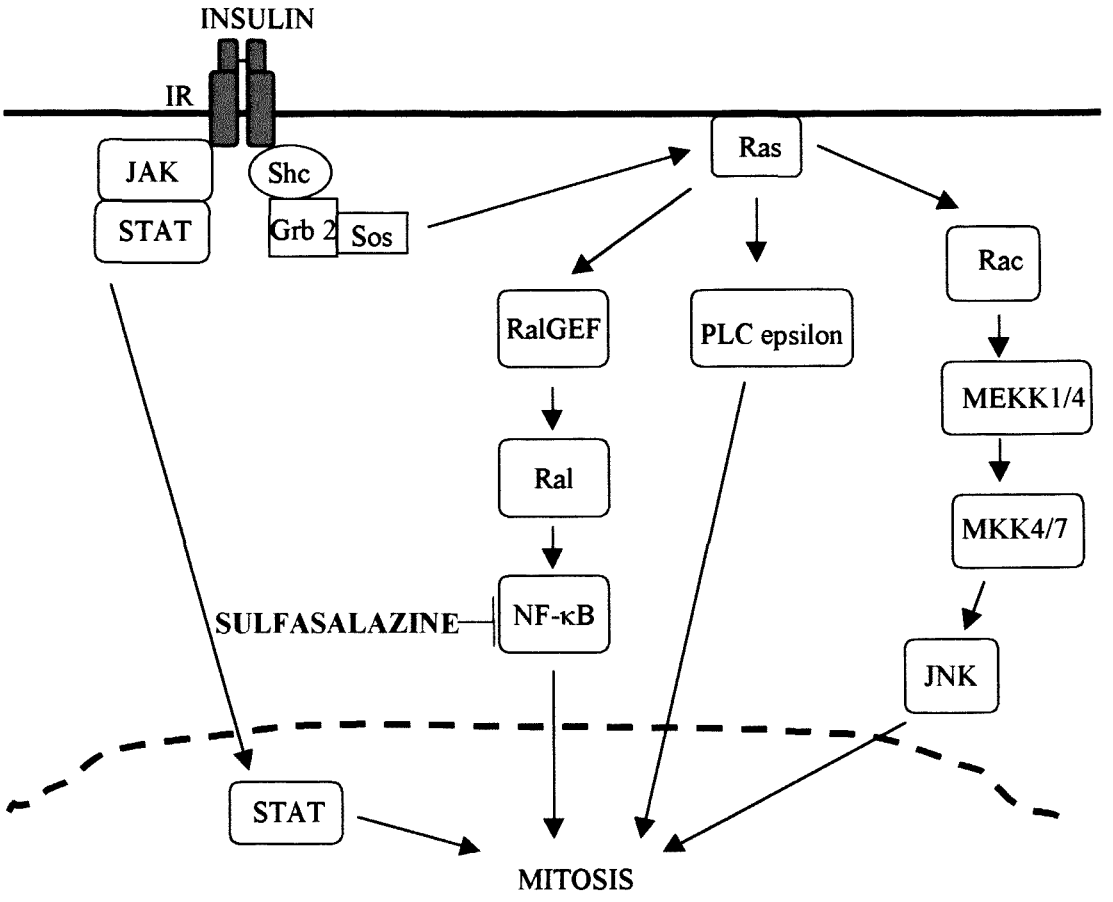

Figure 17: Possible alternative signaling pathways in MBA-15.4 osteoblasts. Ras-dependent pathways include the downstream effectors RalGEF, the recently discovered PLCepsilon and the SAPK, JNK. The Jak/Stat pathway is activated independent of Ras and active Stat translocates to the nucleus.

Alternative pathways

Insulin activated ERK activity in a mouse preosteoblast cell line was sensitive to both MEK and PI3-kinase inhibition. However, proliferation in response to insulin was independent of MEK and PI3-kinase in both young and mature mouse osteoblasts. In contrast, insulin- induced cell proliferation in a human cell line was dependent on both MEK and PI3-kinase. It seems that in MBA-15.4 and MG-63 cell lines, insulin-induced osteoblast proliferation is signaled through different pathways. Since both the MBA-15.4 and 15.6 cell lines used a MEK and PI3-kinase-independent pathway in response to insulin this may indicate a species difference. However, this would have to be confirmed by experiments in primary culture of mouse and human osteoblasts. Also the unique finding that, in mouse cells activation, of both MEK and PI3-kinase is not required for proliferation indicated that a different or even completely novel pathway may be involved in insulin induced-proliferation in mouse bone cells.

Ras-Ral

Ras signals to the cell cycle through Raf and PI3-kinase, but a third effector pathway is also frequently involved (Fig.17). Ral guanine nucleotide exchange factors (GEFs) are classified as the third class of Ras effectors that is functional *in vivo.* In A14-NIH3T3 cells, insulin induced activation of Ral is blocked by a dominant negative Ras, providing evidence that the RalGEF-Ral pathway is downstream of Ras and involve in insulin signaling (Wolthuis et al, 1998). RalGEFs activate Ral, a Ras-like small GTPase, by catalyzing the exchange of GDP for GTP. The Ral isoforms RalA and RalB are 85% identical and 50% identical to Ras. The four known GEFs for Ral includes RalGDS, Rgl, Rlf and Rgr (Wolthuis and Bos, 1999). The RalGDS-related protein Rgr forms part of the fusion protein Rsc (D'Adamo et al, 1997). The RalGEF contains a carboxy-terminal Ras binding domain for binding to active or GTP-bound Ras. An increase in intracellular calcium levels can cause activation of the RalGEFs independent of Ras-binding (Hofer et al, 1998). Ral is present in cytoplasmic vesicles and at the plasma membrane. Activation of RalGEFs involves binding of Ras and induction of a conformational change of the RBD and recruitment to Ral at the plasma membrane. Proteins that have been identified as possible Ral downstream effectors, include phospholipase D1 (PLD1) (Jiang et al, 1995), Ral-binding protein1 (RalBP1) (Cantor et al, 1995) and filamin (Ohta et al, 1999). Other Ral-interacting proteins include NF-κB which regulates cyclin D1 transcription and activation, and Ral-mediated phosphorylation of the transcription factors c-Jun and AFX (Henry et al, 2000). In addition, insulin-induced c-Jun phosphorylation requires Ras, Ral and c-jun NH2 terminal kinase (JNK) activation, a still elusive JNKK and possibly Src (De Ruiter et al, 2000). Using sulfasalazine, a NF-κB inhibitor, we were unable to show inhibition of insulin-induced proliferation with various

concentrations of the inhibitor, indicating that NF-κB may not be involved as an alternative pathway in the MBA-15.4 preosteoblasts (results not shown). Preston et al., (1997) similarly found that sulfasalazine had no inhibitory effect on the more mature UMR-106 rat cells after 24 or 48 h proliferation at any non-toxic concentration. Identification of the Ral downstream targets are important for understanding this pathway labeled as the third Ras effector that has been implicated in insulin signaling.

Ras-PLCepsilon

Another pathway that may be involve is the recently identified and novel fourth class of mammalian phosphoinositide-specific phospholipases, phospholipase C epsilon (PLCε) (Kelley et al, 2001). PLCepsilon contains an amino-terminal RasGTP exchange factor domain and two carboxy-terminal Ras-association domains, RA1 and RA2. PLCepsilon can bind to activated Ras via its RA2 domain, while RA1 binds to inactive Ras with low affinity (Cullen, 2001). However, the mechanism of PLCepsilon activation by Ras is still unclear and may involve translocation to the plasma membrane and activation via the RA1 and RA2 tandem domains (Fig.17).

Ras-JNK

Apart from ERK activation, Ras can also activate the stress-activated protein kinases (SAPK), JNK and p-38. Activation of JNK by insulin has been demonstrated in studies overexpressing human insulin receptors. In Rat 1 fibroblasts insulin-stimulated JNK activity and insulin-induced AP-1 transcriptional activity were found to be Ras-dependent (Miller et al, 1996). In Chinese hamster ovary cells (CHO) acute insulin treatment induced a time-dependent increase in both in JNK and ERK activity. However, prolonged insulin exposure markedly inhibited JNK activity while inducing sustained ERK activation (Desbois-Mouthon et al, 1998). This study indicated that insulin differentially regulates JNK and ERK and provided evidence that insulin exerts opposite effects on JNK activity according to the duration of treatment (Fig.17).

Ras-independent signaling via JAK-STATS

The Janus kinase (Jak) and signal-transducer and activator of transcription (Stat) are necessary components of cytokine receptor signaling. Receptor bound Stat are phosphorylated by Jaks, dissociate from the receptor, dimerize and translocate to the nucleus (Fig.17). Chen et al., (1997) show that Stat5B is a direct substrate of the insulin receptor without the involvement of Jak. Insulin induces tyrosine phsophorylation of Stat5B, which leads to its activation and translocation to the nucleus.

Insulin can also activate Stat3, independently of Ras-ERK and PI3-kinase signal transduction (Coffer et al, 1997). Also suppressor of cytokine signaling (SOCS) -3, a negative regulator restricted to activators of the cytokine receptor family, has been shown to be expressed by interaction with the insulin receptor in 3T3-L1 adipocytes (Emaneulli et al, 2000). Once induced, SOCS-3 inhibits insulin activation of Stat5B without modifying the insulin receptor tyrosine kinase activity. Firstly, from these studies it seems that insulin can induce gene transcription by a signaling cascade distinct from the classical ERK and PI3-kinase pathways. Secondly, a direct signaling path may exist involving only the insulin receptor, Stat5B or Stat3 and the nucleus. However, tyrosine phosphorylation of the Jak proteins induced by insulin has been illustrated indicating possible involvement of Jak proteins upstream of Stats in insulin signal transduction (Giorgetti-Peraldi et al, 1995; Saad et al, 1996). Furthermore, SOCS-1 negatively regulates insulin signaling through suppression of IRS-1 phosphorylation and inhibition of Jak activation (Kawazoe et al, 2001).

In conclusion, we were able to show that insulin does play a role as an osteoblast mitogen. Three immature bone cell lines responded with a marked increase in osteoblast proliferation, compared to a much weaker response by the differentiated UMR-106 rat cell line. In the immature mouse MBA15.4, 20% FCS-induced DNA synthesis was completely dependent on the mitogenic Raf-MEK-ERK signaling pathway, whereas insulin did not rely on PI3-kinase or the Raf-MEK-ERK kinase cascade to signal proliferation. In an immature human cell line MG-63, the phorbol ester TPA only partially depended on PI3-kinase, and it seems as if MEK-ERK was the dominant pathway used for proliferation. Also, in contrast to the MBA-15.4 osteoblasts, the MG-63s equally depended on PI3-kinase and the Raf-MEK-ERK cascade for DNA synthesis in response to insulin. There are clear differences in the proliferative potential of bone cells in response to insulin, which may be due to differences between immortal cell lines, or even the stage of differentiation of the cells. As with the mitogens, TPA and 20% FCS, steroid treatment negatively affected insulin-stimulated proliferation. The PTP inhibitor, vanadate protects against GC-induced decreases in proliferative response to TPA and FCS. However, vanadate failed to reverse steroid-impaired proliferation in response to insulin. Although we showed association of candidate phosphatases, PTP-1B and SHP-1, with molecules in the kinase cascade, it seems as if they are not important as negative regulators in the insulin signaling pathways in MBA-15.4 mouse osteoblasts. The novel proliferative pathway by which insulin signals in MBA-15.4 and 15.6 osteoblasts remains to be discovered.

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