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Prostaglandin Induction of Icer Expression in Osteoblastic Cells and Mouse Calvariae

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ABSTRACT

Inducible cAMP early repressor (ICER) is an early response gene that is transcribed from an intronic promoter of the CREM gene. ICER and other CREM transcripts are expressed in pineal gland, pituitary gland, sertoli cells, as well as bone and are thought to have a role in the physiology of circadian rhythms and spermatogenesis. ICER proteins consist of the DNA binding domains of CREM and act as dominant negative repressors of gene transcription. Parathyroid hormone induces ICER expression in osteoblastic cells through the cAMP/PKA pathway. Prostaglandins also work through the cAMP/PKA pathway and have a myriad of effects on bone metabolism. To determine whether prostaglandins induce ICER expression in osteoblastic cells, MC3T3-E1 cells and mouse calvariae were treated with 1 μM PGE_2, 1 μM $PGF_{2\alpha}$ and 10 nM PTH. The RNA extracted from these osteoblastic cells and calvariae was analysed for ICER and osteocalcin expression using RT-PCR and/or Northern blot analysis. PGE_2 and $PGF_{2\alpha}$ both induced ICER expression, but $PGF_{2\alpha}$ was a weaker agonist. This induction was maximal at 10 μM PGE_2 and 1 μM $PGF_{2\alpha}$ and returned to basal levels by 0.1 nM. ICER expression was also time dependent with a peak at 2 h that returned to baseline by 10 h. We also studied the relation between cell differentiation and the expression of ICER by treating MC3T3-E1 cells with or without ascorbic acid for up to three weeks. Ascorbic acid was not absolutely necessary for the differentiation of MC3T3-E1 osteoblastic cells. However, induction of ICER increased as MC3T3-E1 cells differentiated with ascorbic acid. We conclude that protaglandins E_2 and $F_{2\alpha}$ induce ICER expression in MC3T3-E1 osteoblastic cells and neonatal mouse calvariae and this induction is greater as the cells differentiate. ICER may play a role in prostaglandin mediated gene expression in bone.

INTRODUCTION

Bone

Bone is the integral part of the skeletal system that serves four functions: (i) mechanical function for soft tissue attachment and movement; (ii) protection for vital organs; (iii) metabolic function by acting as a reservoir of ions; and (iv) production of blood cells by hemotopoeisis. The fundamental constituents of bone are the cells and the extracellular matrix. Bone cells include osteoblasts, osteocytes and osteoclasts (1).

There are two types of bone based on the consistency: cancellous bone, also called spongy or trabecular bone, and cortical or compact bone, also called medullary bone. The marrow in cancellous bone consists of thin trabeculae that are filled with hematopoietic bone marrow. This type of bone fulfills a metabolic function. However, cortical bone is composed of thick and dense calcified tissue which fulfills the mechanical and protective function of bone. Cortical bone has two types of surfaces. The inner surface that faces the bone marrow is called the endosteum. The outer layer that faces the surrounding soft tissue is called periosteum. The endosteum is in intimate contact with blood vessels and the cells lining the endosteum are metabolically active and involved in bone remodeling.

Bone is a composite of organic and inorganic matter. Approximately 70% of bone is mineralized or inorganic matter. The inorganic component of bone is mainly composed of a calcium phosphate mineral similar to calcium hydroxyapetite. The organic bone matrix is composed of collagen fibers, mainly type I collagen, osteocalcin, osteonectin, glycoproteins, proteoglycans, and growth factors. Glycoproteins and proteoglycans are highly anionic complexes that have a high affinity for ions. Hydroxyapetite crystals are found on the collagen fibers as well as in the ground substance.

Osteoblasts are bone forming cells that originate from local mesenchymal stem cells in the bone marrow stromal compartment or connective tissue mesenchymal stem cells (2). These precursors differentiate into preosteoblasts and then into mature osteoblasts. Osteoblasts are characterized by their high metabolic activity. This is evident by the presence of extensive endoplasmic reticulum in the cytoplasm as well as an intricate golgi apparatus. This feature of osteoblastic cells makes it possible for them to produce a large amount of collagen that is secreted from the cells. Another characteristic of osteoblastic cells is the presence of a substantial amount of alkaline phosphatase enzyme, which is involved in the process of mineralization of the bone matrix. Osteoblasts express receptors for various hormones such as parathyroid hormone (PTH), estrogens and vitamin D_3. As osteoblasts secrete bone matrix that becomes calcified, they become trapped into the bone matrix and differentiate into osteocytes. Osteocytes have long cell processes that communicate with other cells through gap junctions. Osteocytes remain metabolically active and play a role in activating bone turnover.

Osteoclasts, the major bone resorbing cells, are derived from hematopoietic cells (3). They are large multinucleated cells that contain 4-20 nuclei depending on the species. Osteoclasts are characterized by primary lysosomes, abundance of golgi complexes, and numerous and pleiomorphic mitochondria. The most important feature of the osteoclast is the presence of folds in the plasma membrane that is in contact with the bone matrix. This area is called the ruffled border and is the site at which resorption takes place. The area surrounding the ruffled border contains actin contractile protein filaments that help in attaching the ruffled border to the bone surface. Osteoclasts are located on the endosteal bone surfaces, occasionally on periosteal surfaces and frequently at sites where active remodeling of bone is occurring.

The process of remodeling in the adult skeleton involves a tight coupling between bone resorption and bone formation (1, 4). Wolff's law states that bone remodels in order to withstand the mechanical stresses exerted upon it (5). Under normal conditions, remodeling causes a reduction in bone mass where there is low or no mechanical loading on bone and formation of new bone where bone is highly loaded. Remodeling starts with a period of activation, followed by bone resorption, reversal phase and finally to bone formation. This is evident by the fact that almost all osteoclastic activity is followed by osteoblastic activity. It is believed that osteoblasts mediate the signals for osteoclastic bone resorption. Therefore, hormonal regulation of bone resorption occurs through osteoblastic cells.

Osteoblastic MC3T3-E1 Cells

MC3T3-E1 cells are derived from newborn murine calvariae and have been reported to exhibit a similar developmental sequence to osteoblasts and display osteoblast-like characteristcis after repeated passages (6). It has also been shown that calcified bone tissue is formed in MC3T3-E1 cell cultures in the same manner seen in intramembraneous osteogenesis in vivo. They have the capacity to differentiate into osteoblasts and deposit hydroxyapetite. Osteoblastic cells go through a developmental sequence that initiates from proliferation of undifferentiated osteoblast precursors followed by expression of differentiated osteoblastic phenotype (7, 8). During the initial phase, immature osteoblastic cells actively replicate. Genes that are required for cell growth are actively expressed. Maximal levels of type I collagen mRNA and DNA synthesis are achieved. In the intermediate phase, cells attain confluence, display a cuboidal morphology and stop growing. The downregulation of replication occurs in conjunction with the occurrence of osteoblastic phenotypic markers. These include the production of alkaline phosphatase, processing of procollagens to collagens, and secretion of extracellular matrix protein. Alkaline phosphatase activity is minimal during active replication but increases significantly

with the onset of growth arrest. Therefore, it is thought to be coupled with the downregulation of replication (9). The final phase of osteoblast phenotypic development is characterized by mineralization of the extracellular matrix (7, 9). In osteoblastic MC3T3-E1 cells, the expression of osteoblast markers follow a temporal sequence with production of type I procollagen mRNA preceeding the induction of alkaline phosphatase, followed by osteocalcin mRNAs. The functional similarities that exist between MC3T3-E1 cells growth and normal osteoblasts support the use of the MC3T3-E1 culture system as a useful model to examine cell growth and differentiation (9).

There is a functional relationship between expression of genes in osteoblasts and events associated with osteoblast phenotypic development. For example, high levels of type I collagen mRNA expression during osteoblast proliferation may indicate that the proliferative period supports the synthesis and deposition of collagen required for an extracellular matrix competent for the ordered deposition of mineral (8). Therefore, it is reasonable to test the effect of ascorbic acid, a requirement for collagen synthesis, on the development of the osteoblastic phenotype. The addition of ascorbic acid to fetal rat calvarial cells increases total collagen accumulation paralleled by higher levels of alkaline phosphatase activity and higher levels of mineral accumulation in the cell layer. In addition, the downregulation of proliferation is reached early with the addition of ascorbic acid (8). Ascorbic acid increases osteocalcin synthesis and the addition of β-glycerolphosphate accelerates the onset of mineralized nodule formation coincident with the increase in osteocalcin expression (7). However, primary osteoblastic cultures are complicated by the presence of various cell populations, such as fibroblasts, chondroblasts. Investigation of osteoblastic function in a clonal cell culture model overcomes this limitation.

The MC3T3-E1 cell line has been used to find a possible association between collagen production and osteoblastic marker expression utilizing ascorbic acid and inhibitors of collagen production (10). Ascorbic acid increases the expression of alkaline phosphatase and osteocalcin in MC3T3-E1 cells. Ascorbic acid also dramatically increases collagen matrix accumulation which forms only after exposure to ascorbic acid. Mineralization of the extracellular matrix was greatly stimulated by ascorbic acid and β-glycerol phosphate. However, β-glycerolphosphate alone did not affect any of the osteoblastic development markers. These osteoblastic markers were not expressed when cells were grown in the absence of ascorbic acid or in presence of collagen synthesis inhibitors. These changes in collagen matrix production are attributed to posttranscriptinal mechanisms that ascorbic acid could modify.

Prostaglandins

Prostaglandins are powerful regulators that have both stimulatory and inhibitory effects on bone metabolism. They have been shown to promote bone resorption (11). Prostaglandins also mediate the bone resorptive action of other inflammatory products such as bradykinins and interleukin-1 (12). Moreover, they play a role in the pathogenesis of bone loss in some diseases. Examples are alveolar bone loss in periodontal disease (13) hypercalcemia and increased bone resorption associated with malignancy (14-16) and chronic inflammation (17). Prostaglandins and collagenase are the only resorbing factors detected in odontogenic cysts (13). Substantial amounts of PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ are released by dental cyst capsule fibroblasts (18). Prostaglandins have also been reported to enhance orthodontic tooth movement when given exogenously. However, this enhancement is independent on the concentration of prostaglandins injected or the number of injections (19). Prostaglandins may also play a role in postmenopausal osteoporosis (20). Prostaglandins, in addition to other cytokines, increase bone resorption induced by ovariectomy in mice. Individually, these cytokines are ineffective in causing increased

bone resorption due to estrogen withdrawal. Bone resorption occurs because of the cumulative effects of these cytokines (21). The bone resorbing effect of prostaglandins has been controversial since some studies have shown that prostaglandins transiently inhibit bone resorption (22). The addition of prostaglandins, mainly PGE_2, to mouse monocyte/UMR106 cocultures greatly inhibits the formation of osteoclast like cells (23). This effect is time and cell type dependent since this inhibition did not occur when PGE_2 was added after differentiation to osteoclast like cells. Furthermore, the addition of PGE_2 to ST2 marrow preadipocytic cells stimulates bone resorption. However, some studies suggest that differences in PGE_2 induced bone resorption/formation may be due to the influence of coexisting osteoblasts (24). Prostaglandins cause a significant increase in the osteoclast-like cell formation. However, this increase only occurs in the presence of osteoblasts and is inhibited by indomethacin (24). Prostaglandins promote bone resorption either by themselves or by acting together with resorptive agents such as cytokines. The PGE series has been shown to be the most potent of prostaglandins in causing bone resorption at a range of concentrations from 10^{-9} to 10^{-5}M. $PGF_{2\alpha}$ is less effective than PGE_2 and it's ability to stimulate bone resorption is dependent on PGE_2 induction. (25).

Stimulation of bone formation by prostaglandins has been demonstrated in organ cultures where this stimulation is increased in the presence of cortisol (25). This is mediated by cAMP and may be associated with increased insulin like growth factor-I expression (26). Mechanical forces can elicit a response in bone cells by stretching, fluid shear stress or impact loading (27). Prostaglandins play a role in skeletal growth in fracture repair and impact loading (28-30). It has been suggested that impact loading causes production of PGE_2 and PGI_2 which leads to increased production of growth factors that stimulates differentiation and replication of osteoblasts. Therefore, impact loading can lead to a prolonged period of increased bone formation (31). Zaman and co-workers found an increase in the production of PGE_2, PGI_2 and glucose 6-phosphate dehydrogenase

activity that was blocked by indomethacin. There is also an increase in PGE_2 release in bone cells that are subjected to strain (32). It has been shown that fluid shear stress increases the intracellular cAMP, PGE_2 and IP_3 (33, 34). Stimulation of bone formation and resorption by prostaglandins involves replication and differentiation of osteoclast and osteoblast precursors and may be partly mediated by cAMP (35).

Prostaglandins are produced by osteoblasts and adjacent cells to the bone marrow. Some stimulators of bone resorption can increase prostaglandin production while inhibitors of bone resorption can prevent it. Many bone resorbing factors mediate their effects through production of prostaglandins. Resorption stimulators include the cytokines IL-1 and TNF, growth factors such as TGF-α and TGF-β, as well as hormones including PTH, PTH related peptide (PTHrP), 1,25 $(OH)_2D_3$, and thyroid hormone. Glucocorticoids, IL-4 and nonsteroidal antiinflammatories inhibit bone resorption and prostaglandin production (36, 37). It has also been reported that prostaglandins can induce their own production by stimulating prostaglandin G/H synthase-2 (PGHS-2). This has been shown in vitro in MC3T3-E1 cells (38, 39).

The first step in prostaglandin synthesis is the release of arachidonic acid from membrane phospholipids by phospholipase A_2 or by phospholipase C and diacylglycerol lipase (Fig. 1). Prostaglandins are made by the conversion of arachidonic acid to prostanoids via prostaglandin G/H synthase (PGHS). This conversion involves a cyclooxygenase reaction that converts arachidonic acid to PGG_2 and a peroxidase reaction that converts PGG_2 to PGH_2. Intracellular isomerases and reductases convert PGH_2 to PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$, and thromboxane A_2 (35, 40). Regulation of prostaglandin production is achieved in two ways. First by differential mobilization of arachidonic acid from membrane phospholipids and second by alteration in PGHS function (40). There are two isozymes for PGHS that are encoded by different genes. PGHS-1 is constitutively

expressed and is little regulated, while PGHS-2 is inducible by multiple factors. PGHS-2 acts in acute responses such as inflammation, whereas PGHS-1 has a housekeeping function by continuously producing prostanoids. The stimulators of prostaglandin production are IL-1, TNF, bradykinin, TGF α and β, as well as parathyroid hormone (PTH), parathyroid hormone-related peptide (PTH-rP), 1,25 $(OH)_2$ D_3, and thyroid hormone (12, 37, 41, 42). These hormones and cytokines exert their action by increasing the expression of PGHS-2 and by stimulating the release of arachidonic acid. Interleukin-4 and glucocorticoids are inhibitors of prostaglandin production. They act by inhibiting PGHS-2 or by decreasing arachidonic acid release (41). Finally, sex hormones decrease PTH and IL-1 stimulated prostaglandin production (20, 43). Prostaglandins can also amplify their own production by inducing PGHS-2 (38, 41). This effect is greater with $PGF_{2\alpha}$ and PGD_2 than with PGE_2. Unlike most signalling molecules, prostaglandins are not stored but are continuously released to the cell exterior as soon as they are produced. They affect the cells that produce them and the cells in their immediate surroundings. Therefore prostaglandins are considered autocrine and paracrine stimulators.

Prostaglandins are secreted from their producing cells and bind cell surface receptors. There are different prostaglandin receptors on different tissues such as liver, smooth muscle, fat cells, corpus luteum, leukocytes, platelets, and brain. The biological activities mediated by prostaglandins binding to these receptors include contraction and relaxation of smooth muscle, inhibition of gastric acid secretion, inhibition of inflammatory mediator release, immunoregulation, inhibition of autonomic neurotransmitter release, inhibition of platelet aggregation, luteolysis, and myometrial contraction. There is a specific receptor for each of the prostaglandins: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 bind to the DP, EP, FP, IP, and TP receptors, respectively. There are at least four EP receptor subtypes (EP1, EP2, EP3 and EP4) and four EP3 receptor subtypes (EP3A, EP3B, EPC, and EPD). All these receptors have similar structural characteristics including

seven transmembrane domains, but different ligand specificties and signal transduction pathways (44). Each of these receptors elicits responses by coupling with G proteins and activating secondary messenger systems. Intracellular second messengers are Ca^{2+} and cAMP depending on the G protein coupled to the PG receptor. All prostaglandin receptors are considered to be functional receptors since they have a role in autoregulation and their binding site is open to modulation by ligands (44). EP2 and EP4 receptors stimulate cAMP production and function through the activation of adenylate cyclase to mediate an anabolic effect (45).

cAMP Mediated Signal Transduction

Signal transduction starts by binding of a ligand (i.e. hormone or cytokine) to it's receptor (Fig. 2). Ligand binding causes conformational changes in it's receptor and activates GTP-proteins (G_i, G_s or G_q) that transmit a signal to adenylate cyclase or phopholipase C. Multiple signal transduction mechanisms have been described for prostaglandins in osteoblastic cells. This leads to the production of second messangers. These include calcium entry, IP3 and DAG release by PLC, DAG release by PLD, as well as cAMP responses in the osteoblastic cells. The two major signal transduction pathways, protein kinases A (PKA) and C (PKC), are activated primarily by cAMP and diacylglycerol (DAG), respectively. PGE_2 is known to activate PKA and PKC in osteoblast-like cells (46). PGE_2 increases cAMP production via phosphoinosidtide (PI) hydrolysis in osteoblastic-like cells (47), an example of cross-talk (48). $PGF_{2\alpha}$ is also known to activate PKC, PKA as well as the mitogen-activated protein (MAP) kinase pathway through different G proteins (49). $PGF_{2\alpha}$ also induces Ca^{2+} influx from extracellular space via a different pathway that PI hydrolysis is stimulated (50).

In the cAMP/PKA pathway, the binding of a specific ligand will cause the activation of a G protein, which in turn will activate or inhibit adenylate cyclase. Upon

activation, adenylate cyclase produces cAMP, which binds cooperatively to two regulatory sites of PKA, releasing the active catalytic subunit (48). This catalytic subunit translocates to the nucleus where it phosphorylates a number of cytoplasmic and nuclear proteins on a serine residue, for example, position 133 of CREB (51) and position 117 of CREM (52). The phosphorylation of transcriptional factors mediates transcriptional regulation of various genes. The binding of phosphorylated CREB/ATF factors at cAMP-response element (CRE) sites causes transcriptional activation or repression of target genes. The expression of these factors is not induced. They are mainly regulated by differential phosphorylation (53).

CREM

Prostaglandins act by inducing or repressing gene expression in osteoblastic cells. CREM, the cAMP response element modulator, is a member of CREB/ATF family. The transcription of CREM results in multiple proteins by alternative splicing and promoter utilization. These proteins have different DNA binding affinities that act as transcriptional activators and repressors by binding to CREs in the promoter region of genes. CRE is an eight base pair palindromic sequence (TGACGTCA). Many DNA binding proteins, for example CREB, *jun*, *fos,* and C/EBP, have certain features that facilitate binding to DNA (53, 54). All CREB/ATF transcription factors contain the basic domain/leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). Leucine zipper is a coiled sturcture responsible for dimerization and the basic region is involved in recognition and binding to DNA. Dimerization is required for binding to DNA and there is a dimerization code indicating that the zipper regions of all proteins will not dimerize with each other (53). CREM, like other trascriptional factors, exhibits a structural diversity in that it can form homodimers as well as heterodimers. The function of CRE-binding proteins is modulated by phosphorylation by several kinases. CREM is the only member of the CREB/ATF family that is inducible by cAMP. This induction is rapid and does not require protein

synthesis. Upon activation by cAMP mediated binding of transcription factors, there is a rapid increase in the CREM transcription, which peaks after two hours and declines to basal levels by five hours. This feature classifies CREM as an early response gene (55).

As mentioned above, CREM gene has multiple exons that gives rise to activators as well as antagonists of cAMP-inducible transcription. This property is unique to CREM within the CREB/ATF family of transcription factors. This diversity is due to differential splicing (56). The CREM gene encodes two glutamine rich domains (Q1 and Q2) that are responsible for transcriptional activation, a kinase inducible domain or phosphorylation box (P-box) that includes several phosphorylation sites for various kinases, and two DNA binding domains (DBDI and II) (57, 58). The DNA binding domains constitute a leucine zipper and a basic region (55, 59). Upon phosphorylation, the activator protein undergoes a conformational change that exposes the glutamine rich domains, allowing them to interact with the transcription machinery (60). The CREM antagonists α, β and γ lack Q1 and Q2 domains and block cAMP-induced transcription (58). CREMτ is another isoform that includes the glutamine rich domains and acts as a transcriptional activator (56). Upon activation of adenyl cyclase pathway, a serine residue at position 133 of CREB and at position 117 of CREMτ is phophorylated by PKA. The function of glutamine rich domains is activation of transcription. Each glutamine rich domain can function as an activator by itself, as in CREM isoforms τ1 and τ2. The activation of glutamine rich domains are additive on the magnitude of transcriptional activation when both present, with Q2 being more powerful than Q1 (58). Repression occurs either by the binding of nonactivating homodimers or by heterodimerizing with CRE binding activators, thus blocking their activation. Nonactivating homodimers such as CREM α, β and γ compete with transcription activators for binding to CREs and down regulate cAMP-stimulated gene expression.

ICER

The CREM gene also encodes a group of small repressors. The CREM gene includes two promoters, P1 and P2 (59). P2 is an intronic promoter that that directs the transcription of ICER (inducible cAMP early repressor). This intronic promoter is located between the second glutamine rich domain and the γ exon (59). ICER is the only inducible product of CREM because it is initiated at the intronic promoter P2 that contains four CREs in tandem. Therefore, ICER is strongly inducible by cAMP (61). This feature of ICER gene renders it's expression to be rapid and transient. ICER expression does not reuire de novo protein synthesis (55, 62). The expression pattern of ICER shows that CREM belongs to the class of the immediate early response genes. ICER is a small protein of 120 amino acids with a predicted molecular weight of about 13 KDa. Due to alternative splicing, ICER transcripts yield four isoforms: I, Iγ, II, and IIγ. All ICER isoforms lack the Q and kinase inducible domains of CREM but include either DBDI or DBD II which contain the leucine-zipper and basic region. ICER I and II isoforms include the CREM γ domain while the ICER Iγ and IIγ do not (59). Therefore, ICER can bind to DNA but will not activate transcription. Also, ICER can heterodimerize with CREMτ and other CREM proteins. This would indicate that ICER prevents transcription by occupying the CRE sites as inactive homodimers or by dimerizing with activators and blocking them by the formation of non-functional heterodimers. Hence it is a more potent repressor than CREMα or CREMβ. ICER induction is thought to play a role in the transient nature of cAMP-induced gene expression. The kinetics of ICER induction are characteristic of an immediate early gene. Following cAMP stimulus, there is a rapid rise in CREM transcript that peaks at 2-4 hours followed by a rapid down regulation which is delayed by cycloheximide, an inhibitor of protein synthesis (55). The prolonged inducibility by cycloheximide of cAMP-stimulated gene expression suggests that a de novo synthesized protein, such as ICER, may be responsible for attenuating transcription. The presence of

an inducible repressor in a particular cell type or physiological situation could indicate that there is a mechanism for attenuation of gene expression after activation by cAMP (63).

CREM proteins play an important role in the physiology of the pituitary gland, spermatogenesis, circadian rhythms, and in the molecular basis of memory. Circadian rhythms, 24 hour oscillations in an organism's physiology, in mammals can be seen in the pineal gland, where the expression of CREM gradually increases at night reaching a peak at around 2 am, and decreases to basal low level 3 hours after dark-light transition. (59). A hypothalamic circadian clock is located in the suprachiasmatic nucleus (SCN). The adrenergic stimuli originated from suprachiasmatic nucleus are responsible for the elevated expression of CREM in the pinealocytes at night (64). Moreover, CREM transcripts are abundant in testes. Prepubertal testes mainly express repressor forms of CREM while adult testes express the activator form (56). This developmental switch is regulated through a neuroendocrine axis by follicle stimulating hormone (65). The neuroendocrine axis of CREM expression has been shown in hypophysectomized mice (65). The removal of the pituitary gland diminishes the expression of CREMτ in the testis. The tissue specific expression of CREM is indicative of it's important role in regulation of the cAMP responses (57). The fact that ICER is an inducible repressor and may play a role in the autoregulation of CREM shows the rhythmicity of this gene which is a hallmark of a circadian gene expression. ICER expression has been shown in tissues of neuroendocrine origin such as pineal, pituitary, and adrenal glands (59), as well as non-neuroendocrine tissues such as rat brain (66) and T-lymphocytes (67). ICER accounts for rapid decrease in tyrosine hydroxylase enzyme that catalzes the first and rate-limiting step in catecholamine biosynthesis. This enzyme is located in the central nervous system, sympathetic nervous system and the neuroendocrine cells of the adrenal medulla of the adult mammals (68).

Figure 1 Diagrammatic representation of PG biosynthesis. This figure was adapted from Smith (40).

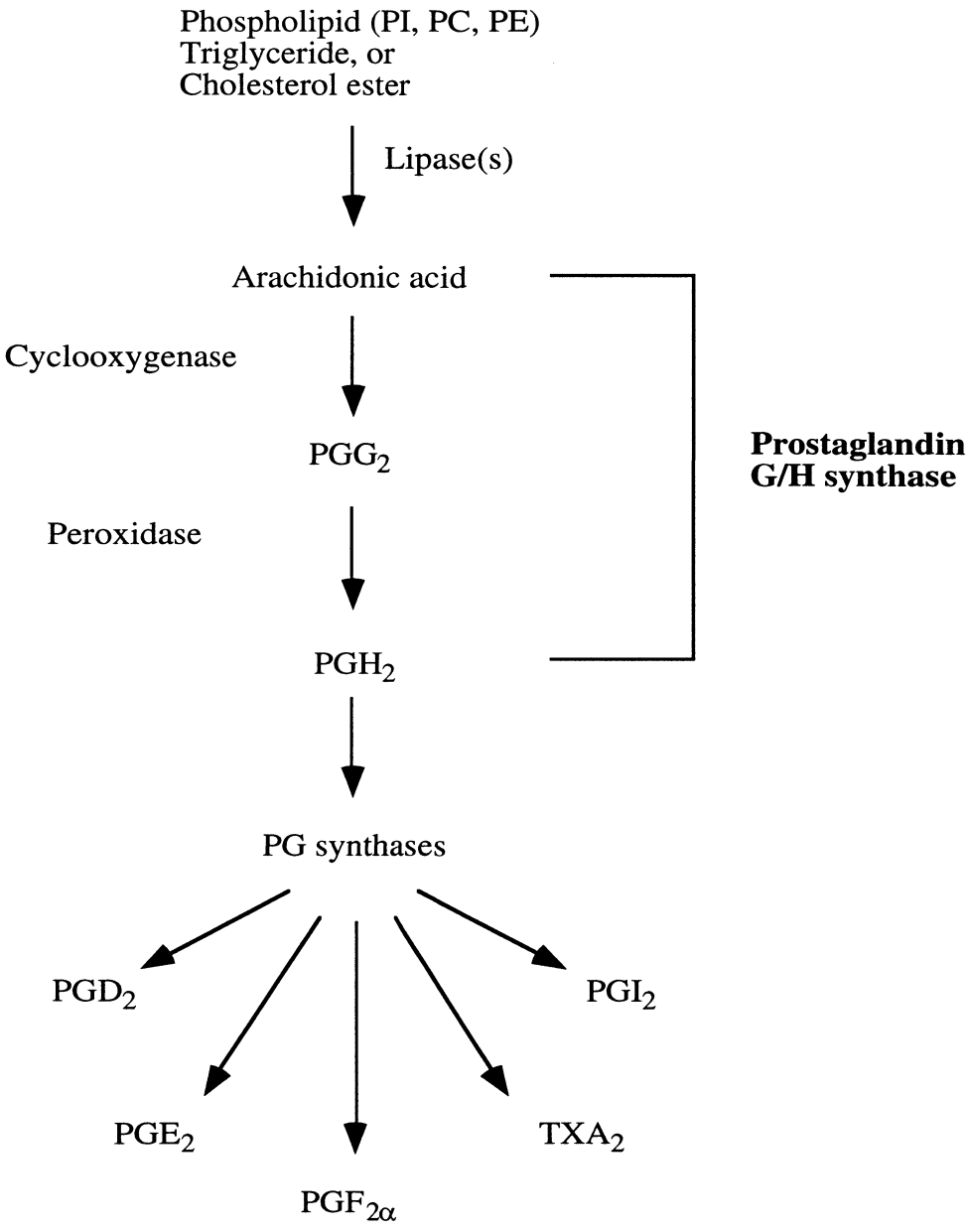
Phospholipid (PI, PC, PE)
Triglyceride, or
Cholesterol ester
Lipase(s)
Arachidonic acid
Cyclooxygenase
PGG2
Peroxidase
PGH2
Prostaglandin
G/H synthase
PG synthases
PGD2
PGI2
PGE2
TXA2
PGF2α

Figure 1

Figure 2 PGE_2 signal transduction pathway through the cAMP-PKA pathway.

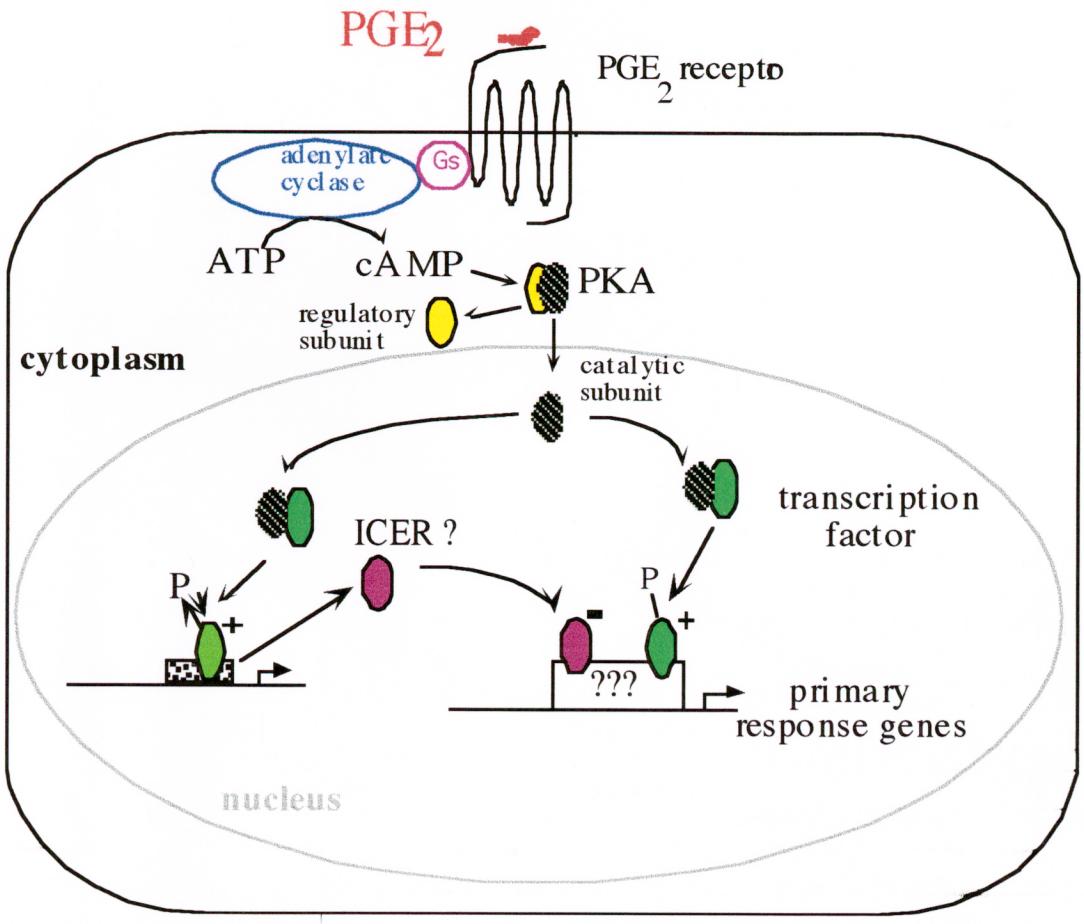
PGE2
PGE2 receptor
adenylate cyclase
Gs
ATP
cAMP
PKA
regulatory subunit
cytoplasm
catalytic subunit
transcription factor
ICER ?
P
P
???
primary response genes
nucleus

Figure 2

HYPOTHESES

PTH induces ICER in osteoblastic MC3T3-E1 cells and cultured neonatal mouse calvariae through the cAMP/PKA pathway (62). Prostaglandins have similar functions to PTH. PGE_2, the most potent bone resorbing prostaglandin is also linked to the PKA signaling pathway in mouse bone cell cultures (24). Also, it has been observed that MC3T3-E1 cells respond differently to a signal depending on their state of differentiation. Therefore, we hypothesize that

- Prostaglandins, which signal through the cAMP-PKA pathway, will transiently induce ICER in MC3T3-E1 cells and neonatal mouse calvariae
- Induction of ICER by prostaglandins will vary with the differentiation state of the cells.

SPECIFIC AIMS

1. To determine if prostaglandins can induce ICER mRNA expression in MC3T3-E1 cells and neonatal mouse calvariae.

   Confluent MC3T3-E1 cells and calvariae from seven day old mice will be treated with 10 nM PTH or various concentrations of PGE_2, or $PGF_{2\alpha}$ for 2 hours. Total RNA will be extracted from one plate of cells or three hemicalvariae for treatment group and quantified at 260 and 280 nm. 3 μg of RNA will be used for reverse transcription (RT). The RT product will be amplified by polymerase chain reaction (PCR) using primers for ICER, GAPDH, and osteocalcin. ICER induction will also measured by Northern blot analysis. RNA will be electrophoresed on an agarose gel, then transferred and immobilized onto a nylon membrane. The immobilized RNA will be probed for ICER, osteocalcin, and actin mRNAs.

2. To determine the time course of ICER induction by prostaglandins in MC3T3-E1 cells.

   Confluent MC3T3-E1 cells will be treated with 1 μM M PGE_2 for 1, 2, 4, 6, and 10 hours. Total RNA will be extracted and quantitated at 260 and 280 nm. ICER, osteocalcin, actin and glyceraldehyde phosphate dehydrogenase (GAPDH) will be measured by reverse transcriptase PCR (RT-PCR) and/or Northern analysis.

3. To determine if the induction of ICER is dependent on the differentiation state of MC3T3-E1 cells.

Confluent MC3T3-E1 cells will be grown with or without ascorbic acid for 1 to 3 weeks and treated with PTH, PGE_2, and $PGF_{2\alpha}$ for two hours. Total RNA will be extracted and quantitated at 260 and 280 nm. ICER, osteocalcin and glyceraldehyde phosphate dehydrogenase (GAPDH) will be measured by reverse transcriptase PCR (RT-PCR).

MATERIALS AND METHODS

Materials

PGE_2 and $PGF_{2\alpha}$ were purchased from Sigma Chemical Company, St. Louis, MO and prepared as a 0.01 M stock solution in ethanol. They were diluted in culture medium to the appropriate concentration. Bovine PTH (1-34) was purchased from Sigma Chemical Company (St. Louis, MO). A 0.1 mM PTH stock solution was prepared in a vehicle containing 1 mg/ml bovine serum albumin (BSA) and 0.001 N HCl. PTH was then diluted in medium at least 1000 fold. L-Ascorbic acid phosphate magnesium salt n-hydrate (phosphoascorbate) was purchased from Wako Pure Chemical Industries, LTD and used in cultures at 50 μg/ml. ICER cDNA was generated by cloning a 169 bp PCR product into the TA vector. Rat osteocalcin (BGP) cDNA was the kind gift of Dr. Vicki Rosen, Genetics, Institute, Cambridge, MA. Chick β-actin cDNA was kindly provided by Dr. Donald Cleveland (Johns Hopkins University, Baltimore, MD).

Cell Culture

MC3T3-E1 cells were plated at 5000 cells/cm^2 in 100 mm culture dishes (Costar, Cambridge, MA), and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) containing 10% heat inactivated fetal calf serum (FCS; Gibco, Grand Island, NY), penicillin (100U/ml), and streptomycin (50 mg/ml). The cells were grown in a humidified incubator in an atomosphere of 5% CO_2, and 95% air at 37°C. The medium was changed every three to four days and the cells were grown to confluence. The cells were treated on the sixth day.

Calvariae

Timed-pregnant female CD-1 mice were obtained from the Charles River Laboratory, Boston, MA. Hemicalvariae of 6-8 day-old offspring were aseptically dissected and precultured for 24 hours in 35 mm plastic culture dishes (Costar, Cambridge, MA) containing 2 ml BGJ medium (Gibco BRL, Grand Island, NY) supplemented with 1 mg/ml BSA, 100 μg/ml ascorbic acid and 1 mM proline for 24 hours prior to hormone treatment.

RNA Extraction

RNA extraction was performed using the method of Chomczynski and Sacchi (69). Pools of six calvariae were homogenized using a polytron (Kinematica, Kriens, LU, Switzerland) in 4M guanidinium isothiocyanate (GTC). Cells were lysed in GTC without homogenization. Following lysis, 0.2ml of sodium acetate, pH 4, 2 ml of water-saturated phenol (Gibco, Grand Island, NY), and 0.5 ml of chloroform-isoamyl alcohol (24:1) were added. Samples were left at 4°C for 15 minutes and centrifuged at 9000 rpm for 20 minutes at 4°C. The aqueous phase was removed, added to one volume of isopropanol and precipitated at -80°C for 15 minutes. The samples were centrifuged again at 9000 rpm for 20 min at 4°C. The pellets were resuspended in 0.3 ml guanidinium isothiocyante. The RNA was precipitated with 1 volume of isopropanol and washed with 80% ethanol. Precipitated RNA was lyophilized and dissolved in 50 μl of water. The RNA was heated at 65°C for 10 minutes. RNA content was quantitated by absorbance at 260 and 280 nm using a Pharmacia Genequant RNA/DNA calculator. The OD_{260} to OD_{280} ratio was used to assess purity of the RNA preparation.

RT-PCR

All materials were obtained from Gibco BRL (Grand Island, NY). 3 μg of RNA was incubated in a 50 μl reaction volume containing oligo dT (1.75 mM final

concentration), 5x buffer, 500 mM dNTPs, 2 mM dithiothreitol, 1U/ml RNase inhibitor (Stratagene, La Jolla, CA), and 13.5 U/ml M-MLV RT enzyme. 5 µl of RT product was incubated with 1.5 mM magnesium chloride, 5 nM primers, 25 U/ml of Taq polymerase, 10X PCR buffer, and water to a final volume of 50 µl. The amplification cycle for ICER consisted of 3 minute denaturation at 94°C for the first cycle and 45 seconds for the remaining cycles, annealing at 65°C for 45 seconds, and extension at 72°C for 2 minutes and 5 minutes at the end. Primers (Gibco BRL) were designed according to the ICER sequence reported by Molina et al. 1993 (55). The 5' primer corresponded to the 5' untranslated region of ICER (5'-TATGCAAAAGCCCAACATGG-3'; primer A, Figure 3) upstream of γ and DNA binding domains. The 3' primer spans nucleotides 1068-1090 downstream of DBD II (5'-CTACTAATCTGTTTTGGGAGAGC-3'; primer C, Figure 3). Murine glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified using specific primers (Clontech Laboratories, Inc., Palo Alto, CA) and served as an internal control. Osteocalcin primers (Gibco BRL, Grand Island, NY) were used to amplify osteocalcin. The same conditions were used for the amplification of GAPDH and osteocalcin with the exception of the annealing temperature which was 60°C for GAPDH and 67°C for osteocalcin. The number of cycles were 25 for ICER and 22 for GAPDH and osteocalcin.

Northern Blot Analysis

A gel containing 1% agarose and 3% formaldehyde was used for Northern blot analysis. 20 µg of RNA from MC3T3-E1 cells or 12.5 µg of RNA from calvariae were lyophilized. Prior to loading on the gel, 0.5 µg of ethidium bromide were added to each sample. The gel ran for three hours on ice and RNA was transferred to a nylon membrane (Genescreen; New England Nuclear Corp., Boston, MA) using positive pressure (Posiblotter, Stratagene, La Jolla, CA). To immobilize the RNA, a UV Stratalinker (Stratagene, La Jolla, CA) was used. The membranes were prehybridized at 42°C for three hours in rotating cylinders (Techne Hybridizer, Princeton, NJ). In addition to the

prehybridization buffer (5prime to 3prime, Boulder, CO), 50% formamide, ss DNA and yeast tRNA (5 prime to 3 prime, inc. Boulder, CO), were added to the prehybridization buffer. Filters were hybridized overnight in hybridization buffer containing 50% formamide, 20% dextran (5prime to 3prime, Boulder, CO), 0.04 mg/ml ssDNA and ytRNA, [^{32}P]dCTP or [^{32}P]dGTP-labled cDNA probes (3000 Ci/mml, New England Nuclear Research Products, DuPont Co, Wilmington, DE) for actin and osteocalcin. The activity of denatured cDNA probes were 2-5 x 10^6 cpm/ml. Both radionucleotides were used to label the ICER probe. Filters were washed once in 1% sodium dodecyl sulfate (SDS)/1X SSC at room temperature for 10 minutes and then heated to 65°C in 0.1% SDS/0.1 X SSC, followed by several washes in 0.1% SDS/0.1 X SSC at room temperature. Filters were exposed to Kodak film with an intensifying screen at -80°C for 1-3 days. Filters were stripped with boiling 0.1% SDS/0.1xSSC between hybridizations.

RESULTS

Our first goal was to determine if prostaglandins could induce ICER mRNA in MC3T3-E1 cells and neonatal mouse calvariae. Confluent MC3T3-E1 cells were treated with PTH at 10 nM and PGE_2 and $PGF_{2\alpha}$ at 1 μM for 2 h. ICER mRNA levels were determined by RT-PCR using primers A and C (Fig. 3). PGE_2 and $PGF_{2\alpha}$ induced ICER mRNA. (Fig. 4) The induction of ICER by these prostanoids was not as strong as by PTH at 10 nM. $PGF_{2\alpha}$ was less effective than PGE_2 or PTH. PTH and PGE_2 induced all ICER isoforms as determined by RT-PCR using primers A and C. In all cases four bands consistent with the predicted sizes of 779, 743, 382, and 346 base pairs were seen. These corresponded to the ICER isoforms I, Iγ, II and IIγ (62). Expression of GAPDH was similar in all samples.

To determine the dose response relation for prostaglandin induction of ICER, MC3T3-E1 cells were treated with various concentrations of PGE_2 and $PGF_{2\alpha}$ for 2 h and ICER mRNA levels were determined by RT-PCR. The maximal induction of ICER was at 10 μM PGE_2. ICER mRNA levels returned to basal levels at 0.1 nM PGE_2 (Fig. 5). Induction by $PGF_{2\alpha}$ at 1 μM was lower than at 100 nM in MC3T3-E1 cells. (Fig. 6). $PGF_{2\alpha}$ had a maximal effect at 100 nM and returned to basal levels at 0.1 nM. Expression of GAPDH and osteocalcin was similar in all samples.

To determine whether prostaglandins and PTH induced ICER expression in bone tissue, neonatal mouse calvariae were treated with PGE_2 and $PGF_{2\alpha}$ at 1 μM and PTH at 10 nM for 2 h. ICER mRNA levels were determined by Northern blot analysis and RT-PCR using primers A and C. All three agonists induced ICER mRNA in neonatal mouse calvariae at 2 h. $PGF_{2\alpha}$ was less effective than PGE_2 or PTH. (Fig. 7a, 7b) A dose response relation was also determined for PGE_2 induction of ICER in neonatal mouse

calvariae at 2 h. PGE_2 induction of ICER was dose dependent. The response was maximal at 1 μM and decreased to low levels at 1 nM. (Fig. 8). Expression of GAPDH and osteocalcin were similar in all samples.

To determine the time course of ICER induction by prostaglandins, MC3T3-E1 cells were treated with 1 μM PGE_2 for 1, 2, 4, 6 and 10 h (Fig. 9). RNA was subjected to RT-PCR using primers A and C. The induction of ICER reached a maximum level at 2 h and returned to baseline by 6 h. ICER induction by PGE_2 in MC3T3-E1 cells was also detectable by Northern analysis, but only at 1 μM. Expression of GAPDH mRNA was equivalent in all samples.

To determine if the induction of ICER by prostaglandins was dependent on the differentiation state of MC3T3-E1 cells, cells were cultured in the presence or absence of 50 μg/ml phosphoascorbate for up to three weeks. After 1, 2 and 3 weeks of culture cells were treated with PTH and prostanoids for 2 h and analyzed for ICER mRNA levels by RT-PCR. At week 1, there was no induction of ICER by PTH or prostanoids in the presence or absence of phosphoascorbate (Fig. 10). At week 2, PTH and prostanoids induced ICER in cells grown with and without ascorbate. However, there was a marked increase in ICER induction by PTH in cells cultured in the presence of phosphoascorbate (Fig. 11a and 11b). Prostaglandins showed a slight enhancement of ICER induction in the presence of phosphoascorbate. At week 3, all agonists showed a higher induction of ICER in the presence or absence of phosphoascorbate (Fig 12a and 12b). The highest ICER levels were induced by PTH, followed by PGE_2 and then $PGF_{2\alpha}$ regardless of the age of the culture or treatment medium. Northern blot analysis of RNA at week 1 showed no detectable osteocalcin production in any of the treatment groups. At weeks 2 and 3, all cells expressed osteocalcin; there was greater osteocalcin mRNA in the phosphoascorbic treated cells. Analysis of the week 2 and 3 Northern blots by densitometry demonstrated

that all ascorbate treated samples had enhanced osteocalcin expression. It is also evident that prostanoids decreased the expression of osteocalcin compared to PTH and control. ICER was too weak to be detected by Northern blot analysis at any of the time points (data not shown). Expression of GAPDH and osteocalcin was similar in all samples.

DISCUSSION

PGE_2 induced all of the known ICER isoforms in osteoblastic MC3T3-E1 cells. This response was rapid and transient. PGE_2 induction of ICER in MC3T3-E1 cells had a peaked at two hours and then declined to baseline levels by ten hours as determined by RT-PCR. PGE_2 induces it's biological effects mainly through EP receptors. EP2 and EP4 receptors are coupled to adenylate cyclase through G_s (44). This enables PGE_2 to stimulate cAMP production and activate PKA. Since ICER induction is tightly coupled to the cAMP-PKA pathway in osteoblastic cells, it is reasonable to hypothesize that PGE_2 induces ICER via EP2 and/or EP4 receptor binding.

$PGF_{2\alpha}$ induction of ICER was lower than either PTH or PGE_2. The induction of ICER by $PGF_{2\alpha}$ peaked at 100 nM and was lower at 1 μM. The actions of $PGF_{2\alpha}$ are carried out primarily through FP receptors. FP receptors interact with a G_q protein and activate phospholipase C (44). Phospholipase C in turn hydrolyzes phophoinositide (PI) into diacylglycerol (DAG) and inositol triphosphate (IP3). This ultimately leads to an IP3 mediated Ca^{2+} release from intracellular pools and activation of PKC by DAG. It has been shown that $PGF_{2\alpha}$ induces Ca^{2+} influx in a dose dependent manner (50). This study reported that Ca^{2+} influx is significantly stimulated by $PGF_{2\alpha}$ even in the presence of a Ca^{2+} antagonist that was maximal at 10 nM. A concentration of $PGF_{2\alpha}$ above 10 nM caused a less than maximal stimulation. Since $PGF_{2\alpha}$ stimulation of IP3 follows a normal curve, it seems that PI hydrolysis and Ca^{2+} influx are carried out by different pathways. This also may explain the lower induction of ICER by $PGF_{2\alpha}$ at higher concentrations. The induction of ICER may be carried out by a different pathway that is concentration sensitive. For example, it is known that $PGF_{2\alpha}$ is partially capable of inducing a response through PKA. The lower level of induction of ICER can also be explained by this partial use of PKA by $PGF_{2\alpha}$. It can also be possible that PKC and PKA pathways converge at the level

of transcriptional activity in MC3T3-E1 cells (38). It would be plausible that activation of PKC could also induce CREM but at a lower level. The level of ICER induction in MC3T3-E1 cells could not be consistently detected by Northern Blot analysis. This may be attributed to several factors such as sensitivity of different batches of MC3T3-E1 cells to prostaglandins or the state of differentiation of these cells.

PTH, PGE_2 as well as $PGF_{2\alpha}$ also induced ICER mRNA in neonatal mouse calvariae. ICER induction by PTH was strongest and $PGF_{2\alpha}$ had the weakest response. The mRNA extracted from mouse calvariae was subjected to Northern blot analysis as well as RT-PCR. A classical dose response relationship was not evident on the Northern analysis. However, RT-PCR indicated a dose response pattern with a peak at the highest concentration (1μM) and decreased induction of ICER corresponding to the decrease in the dose of PGE_2.

MC3T3-E1 cells were treated with ascorbic acid to induce differentiation. Osteocalcin mRNA was measured as a marker of mature osteoblasts. The effect of ascorbic acid on the differentiation of MC3T3-E1 cells was not evident at the first week of incubation, as osteocalcin was not expressed. However, cells expressed osteocalcin after two and three weeks of culture as shown by the Northern blot analysis. Cells cultured with ascorbic acid expressed more osteocalcin than cells cultured without ascorbic acid. However, differentiation was not totally dependent on ascorbic acid since cells without ascorbic acid also expressed osteocalcin at weeks 2 and 3. These data indicate that high cell density may also increase differentiation. In studies that measured osteoblast differentiation as measured by alkaline phophatase activity, no change was detected in the onset of alkaline phosphatase activity in presence of ascorbic acid (9). However, ascorbic acid enhanced alkaline phosphatase activity in mature osteoblasts compared to controls. The same study found the regulation of collagen biosynthesis to be independent of ascorbic acid. These

studies indicate that the presence of ascorbic acid is not required for the development and onset of osteoblastic marker expression and that it can affect only some of these markers.

Expression of ICER in ascorbic acid treated cells did not show an increased response compared to controls at first week. ICER mRNA increased in both ascorbic acid treated and control cells in the following weeks. However, the differentiation state of MC3T3-E1 cells did not increase the induction of ICER by different agonists remarkably. This would probably indicate that the induction of ICER by these agonists is not totally dependent on the differentiation state of these cells.

Possible roles of ICER in PG response:

It has been shown in many studies that exogenous prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2, and PGI_2) can induce PGHS-2 mRNA in osteoblastic MC3T3-E1 and Py1a cells which leads to increases in protein and medium PGE_2 (38, 41). Furthermore, this induction occurs through the cAMP/PKA pathway through enhancement of transcription in MC3T3-E1 cells. In MC3T3-E1 cells the decline of PTH stimulated PGHS-2 transcription required de novo protein synthesis (62). It can be hypothesized that the attenuation of the induced PGHS-2 gene transcription may be mediated by a protein such as ICER. This is speculated since the peak of ICER protein corresponds to the decline in PGHS-2 mRNA. Further, it can be hypothesized that the attenuation of transcription of other primary resoponse genes that occur through the PKA pathway may be mediated by ICER.

Exogenous PGE_2 induces the expression of early response genes such as *c-fos*, *c-jun*, *jun*-B and egr-1 in calvariae and tibiae of 5 week old rats (70). *jun* and *fos* are members of a family of protooncogenes whose products form a dimeric protein complex called activator protein-1 (AP-1). AP-1 is a transcriptional factor that mediates the activation of many genes. Weinerb reported that the induction of these early-response

genes is rapid and transient since the mRNA levels return to baseline (70). Moreover, *c-fos* promoter contains several regulatory sequences including CREs that can mediate a cAMP activation of this gene via a PGE_2 signal. Since PGE_2 causes the induction of ICER in osteoblastic cells, it is plausible to assume that the transient response of *jun* and *fos* may be due to transcriptional attenuation by ICER.

It has been shown that prostaglandins influence the differentiation of osteoclast precursors (22, 23). This influence is dependent on the type and dose of the administered prostaglandin as well as the type of cell used. The differentiation of osteoclasts requires the interaction between hematopoietic osteoclast precursor cells and stromal or osteoblastic cells (43). When prostaglandin E is added to murine bone marrow macrophages prior to the addition of stromal cells, osteoclastic differentiation is enhanced (71). This effect was reproduced by 8-bromo-cAMP. Paradoxically, Quinn and colleagues showed that several types of prostaglandins inhibit the osteoclast differentiation of precursor cells (mouse monocyte/UMR106 cocultures) (23). This effect was time dependent since the addition of prostaglandin after the differentiation of these cells greatly reduced this inhibition. PGE_2 was found to be more potent than PGE_1 and $PGF_{2\alpha}$. However, in the same study prostaglandins increased the amount of lacunar bone resorption in another cell line, ST2 preadipocytic cells. They concluded that the effects of prostaglandins on osteoclast differentiation in vitro are highly dependent on the type of bone derived stromal cells supporting this process (23). The addition of PGE_2 increased intracellular cAMP levels in both cell types. Moreover, this cAMP increase is mimicked by the addition of PTH or forskolin. This further raises the possibility of a PKA signal transduction and gene expression which would imply a possible role of ICER in the attenuation of the response. Fuller and Chambers showed that prostaglandins transiently inhibit bone resorption when added to isolated osteoclasts *in vitro* (22). This calcitonin like inhibitory effect is thought to be due to activation of cAMP formation in osteoclasts. Since prostaglandins may

"transiently" inhibit osteoclast function through cAMP, it is plausible to question the cause of this transitory response and whether ICER plays a role in this response.

Prostaglandins have a stimulatory effect on bone formation. This is thought to occur in part through an increase in the production of insulin-like growth factor-I (IGF-I) (26). This hypothesis is supported by the fact that IGF binding protein-3 decreases collagen synthesis in mouse calvarial organ cultures stimulated by cortisol and PGE_2. Forskolin has a similar anabolic effect whereas phorbol ester does not elicit this response suggesting that the cAMP-PKA pathway mediates the increase in IGF-I production. Therefore, it will be interesting to determine the possible role of ICER in regulating PGE_2 stimulated IGF-I production and bone formation in response to PGE_2.

Figure 3 Schematic representation of ICER transcripts and primers used for PCR.

Primers A and C (arrows) were used to identify ICER transcripts in MC3T3-E1 cells and mouse calvariae. Primer A correstponds to the unique untranslated region of ICER. Primer C is located downstream of DBDII. Predicted sizes of the PCR products are indicated on the right.

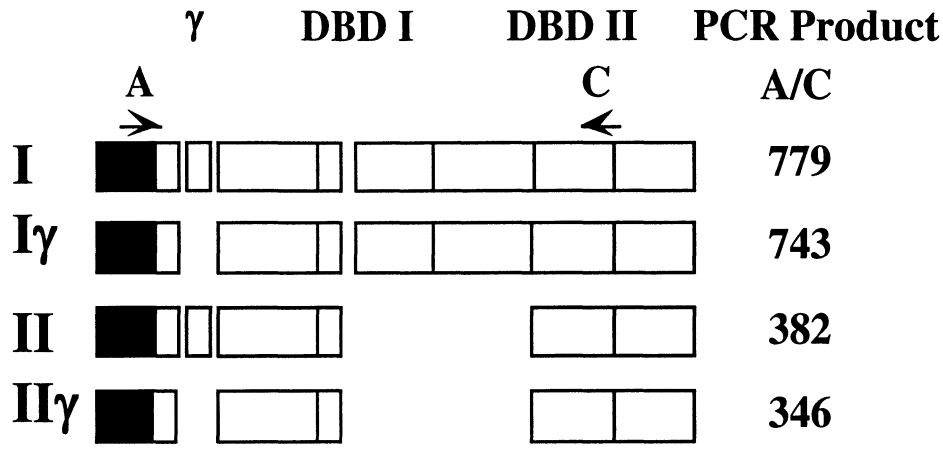

Figure 3

Figure 4 PGE_2 and $PGF_{2\alpha}$ induce ICER mRNA in MC3T3-E1 osteoblastic cells.

Cells were treated with 1 μM PGE_2, 1 μM $PGF_{2\alpha}$ or 1 nM PTH for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

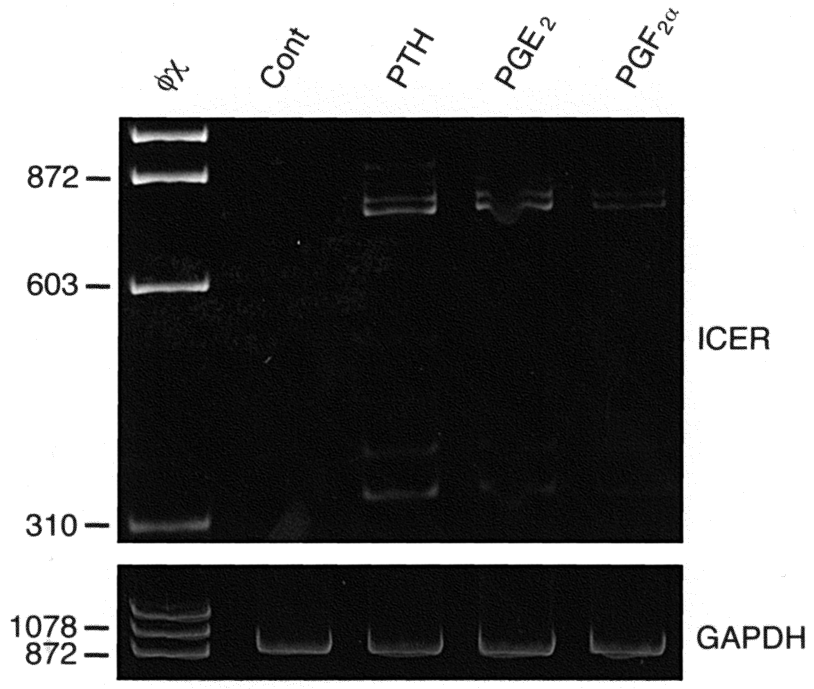

Figure 4

Figure 5 PGE_2 produces a dose-dependent induction of ICER mRNA in MC3T3-E1 osteoblastic cells.

Cells were treated with vehicle, PTH (10 nM) or PGE_2 (0.1 nM to 10 μM) for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

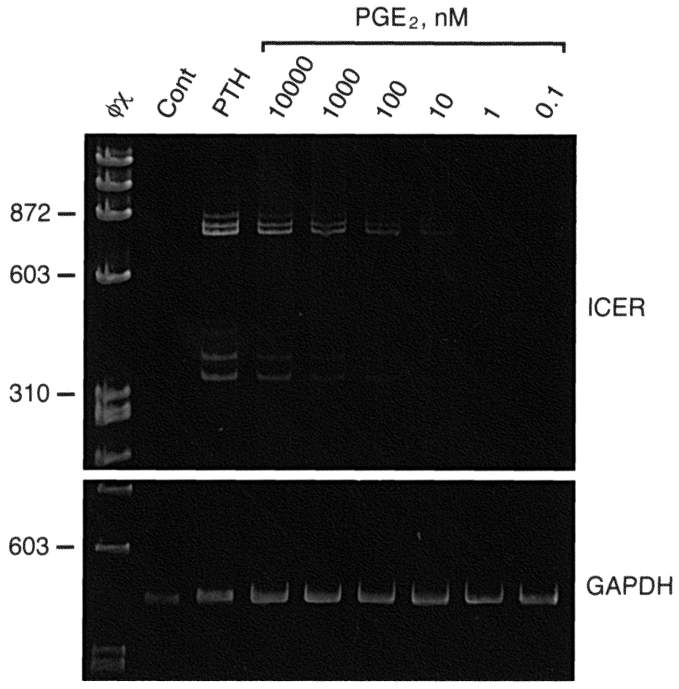
PGE2, nM
φX
Cont
PTH
10000
1000
100
10
1
0.1
872
603
310
ICER
603
GAPDH

Figure 5

Figure 6 $PGF_{2\alpha}$ produces a dose-dependent induction of ICER mRNA in MC3T3-E1 osteoblastic cells.

Cells were treated with vehicle, PTH (10 nM) or $PGF_{2\alpha}$ (0.1 nM to 10 μM) for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

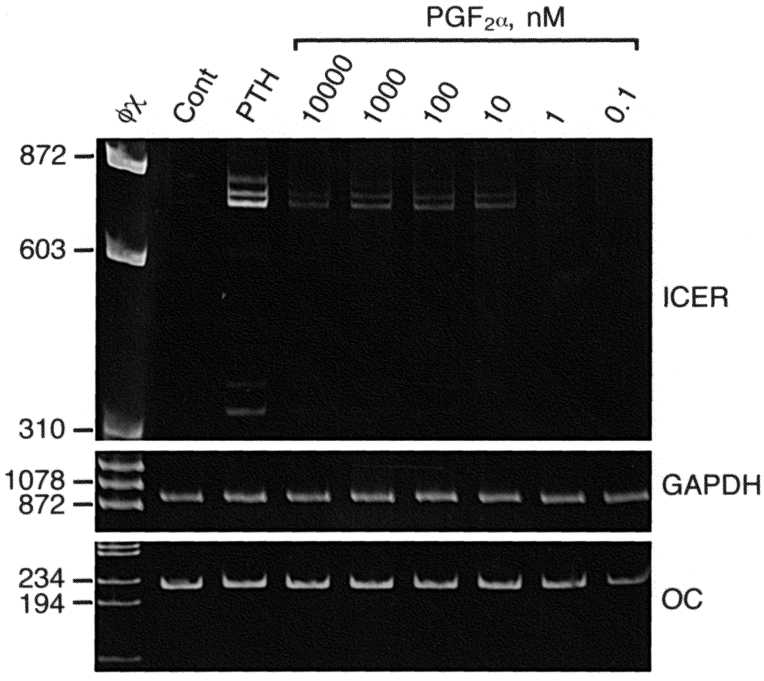
PGF2α, nM
φχ
Cont
PTH
10000
1000
100
10
1
0.1
872
603
310
ICER
1078
872
GAPDH
234
194
OC

Figure 6

Figure 7a PGE_2 and $PGF_{2\alpha}$ induce ICER mRNA in neonatal mouse calvariae.

Calvariae were treated with vehicle, 1 μM PGE_2, 1 μM $PGF_{2\alpha}$ or 1 nM PTH for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

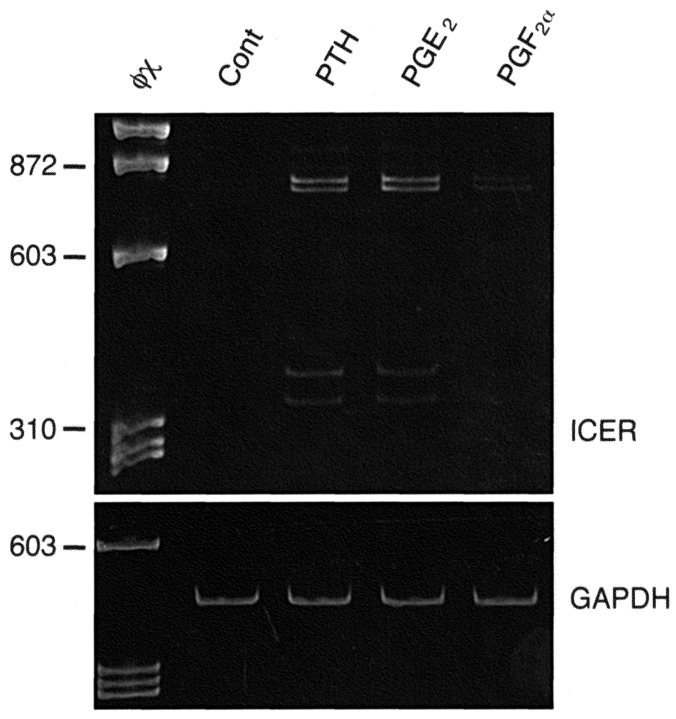

Figure 7a

Figure 7b PGE_2 and $PGF_{2\alpha}$ induce ICER mRNA in neonatal mouse calvariae.

Calvariae were treated with vehicle, 10 nM PTH, 1 μM PGE_2 or 1μM $PGF_{2\alpha}$ for 2 h. RNA was extractred and subjected to Northern blot analysis as described in Materials and Methods.

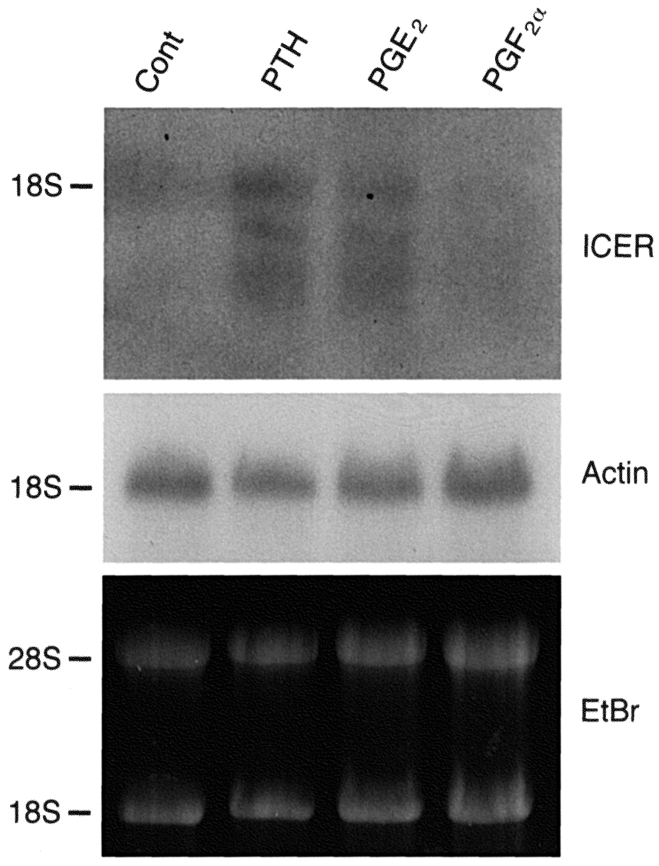

Figure 7b

Figure 8 PGE_2 causes a dose-dependent induction of ICER mRNA in neonatal mouse calvariae.

Calvariae were treated with vehicle, 10 nM PTH, 1 nM to 1 μM PGE_2 for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

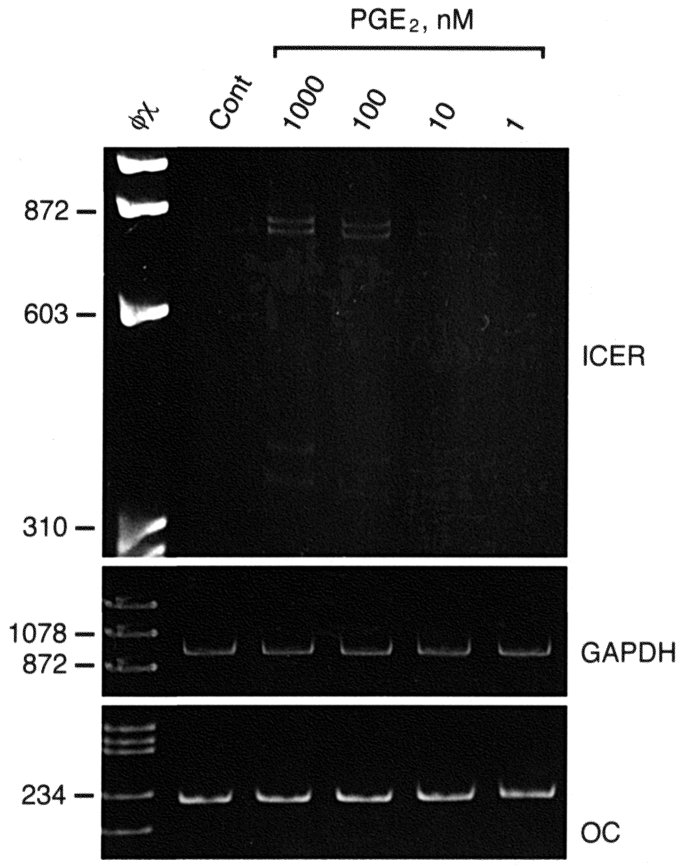
PGE2, nM
φX
Cont
1000
100
10
1
872
603
310
ICER
1078
872
GAPDH
234
OC

Figure 8

Figure 9 PGE_2 produces a time-dependent induction of ICER mRNA in MC3T3-E1 osteoblastic cells.

MC3T3-E1 cells were treated with vehicle or 1 μM PGE_2 for 1 to 10 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

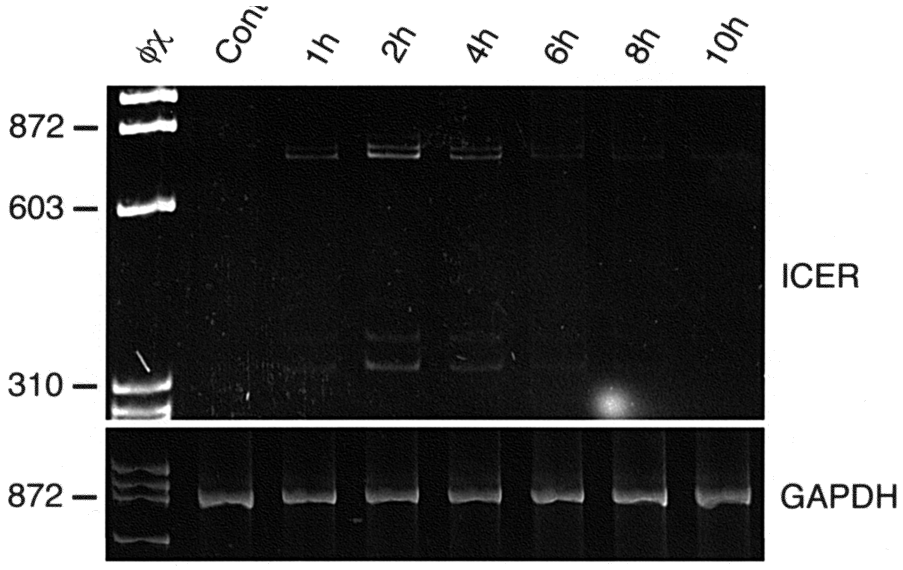

Figure 9

Figure 10 MC3T3-E1 cells cultured for one week express very low ICER and osteocalcin regardless of whether they are grown in the presence or absence of ascorbic acid.

MC3T3-E1 cells were grown in the presence or absence of 50 μg/ml phosphoascorbate for 1 week. Cells were treated with 10 nM PTH, 1 μM PGE_2 or 1 μM $PGF_{2\alpha}$ for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

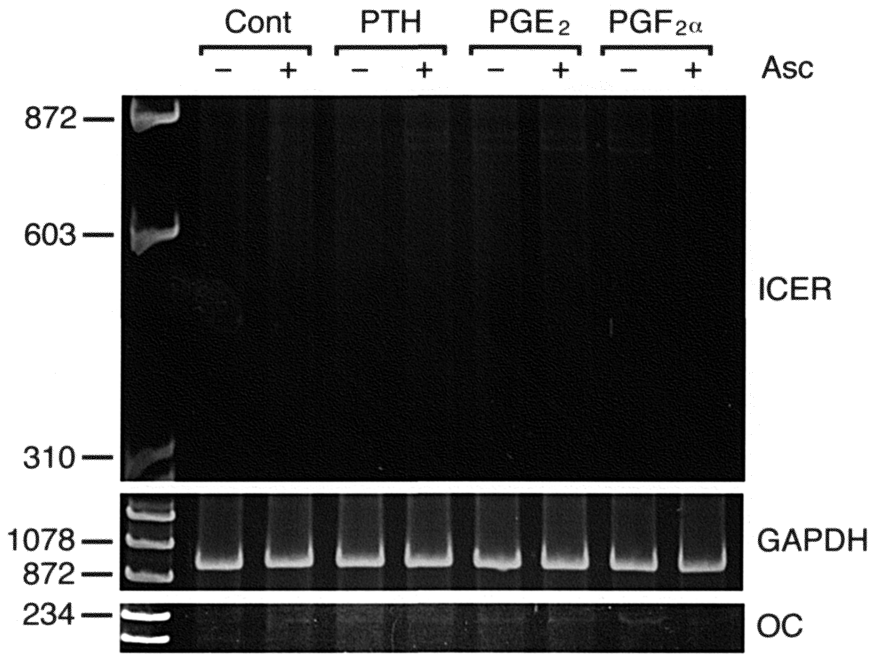

Figure 10

Figure 11a PTH, PGE_2 and $PGF_{2\alpha}$ induce ICER in MC3T3-E1 cells cultured for two weeks in the presence or absence of ascorbic acid.

MC3T3-E1 cells were grown in the presence or absence of 50 μg/ml phosphoascorbate for 2 weeks. Cells were treated with 10 nM PTH, 1 μM PGE_2 or 1 μM $PGF_{2\alpha}$ for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

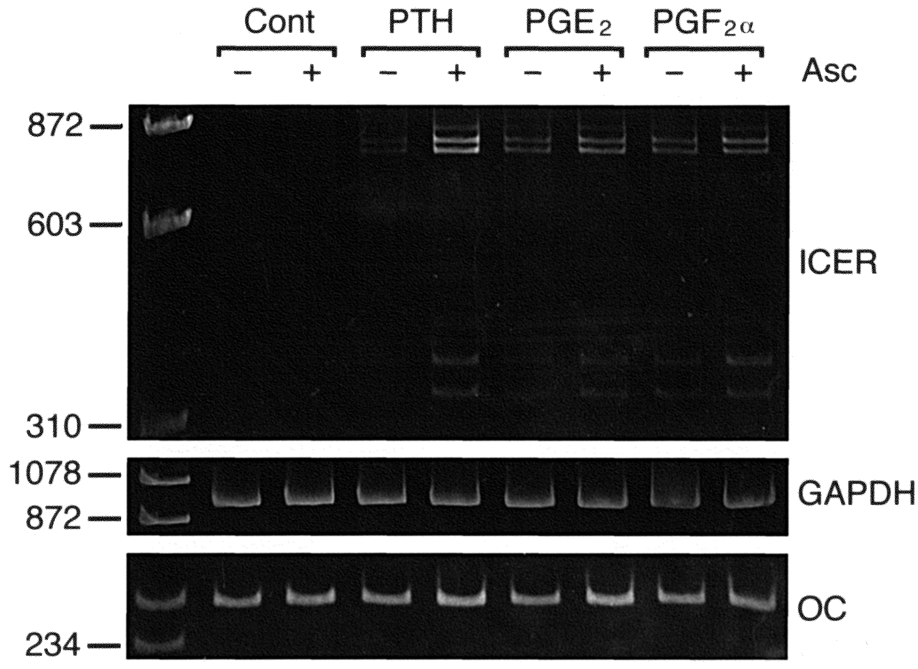

Figure 11a

Figure 11b Osteocalcin expression is higher in ascorbate-treated compared to ascorbate untreated MC3T3-E1 cells cultured for two weeks.

MC3T3-E1 cells were grown in the presence or absence of 50 μg/ml phosphoascorbate for 2 weeks. Cells were treated with 10 nM PTH, 1 μM PGE_2 or $PGF_{2\alpha}$ for 2 h. RNA was extracted and subjected to Northern blot analysis as described in Materials and Methods.

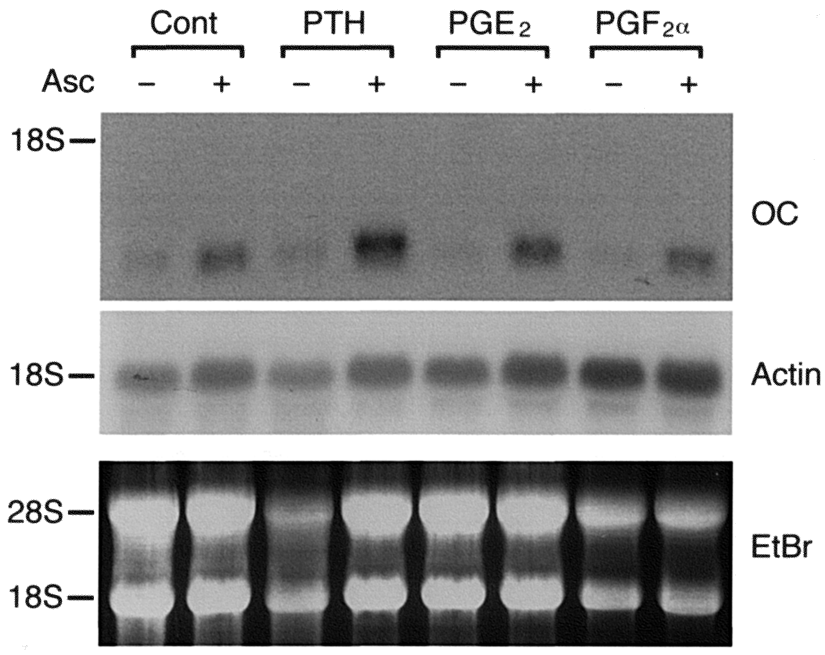

Figure 11b

Figure 11c Densitometry of osteocalcin expression normalized to actin for MC3T3-E1 cells grown for 2 weeks in the presence or absence of phosphoascorbate (Asc). This graph represents densitometric values for Figure 9b.

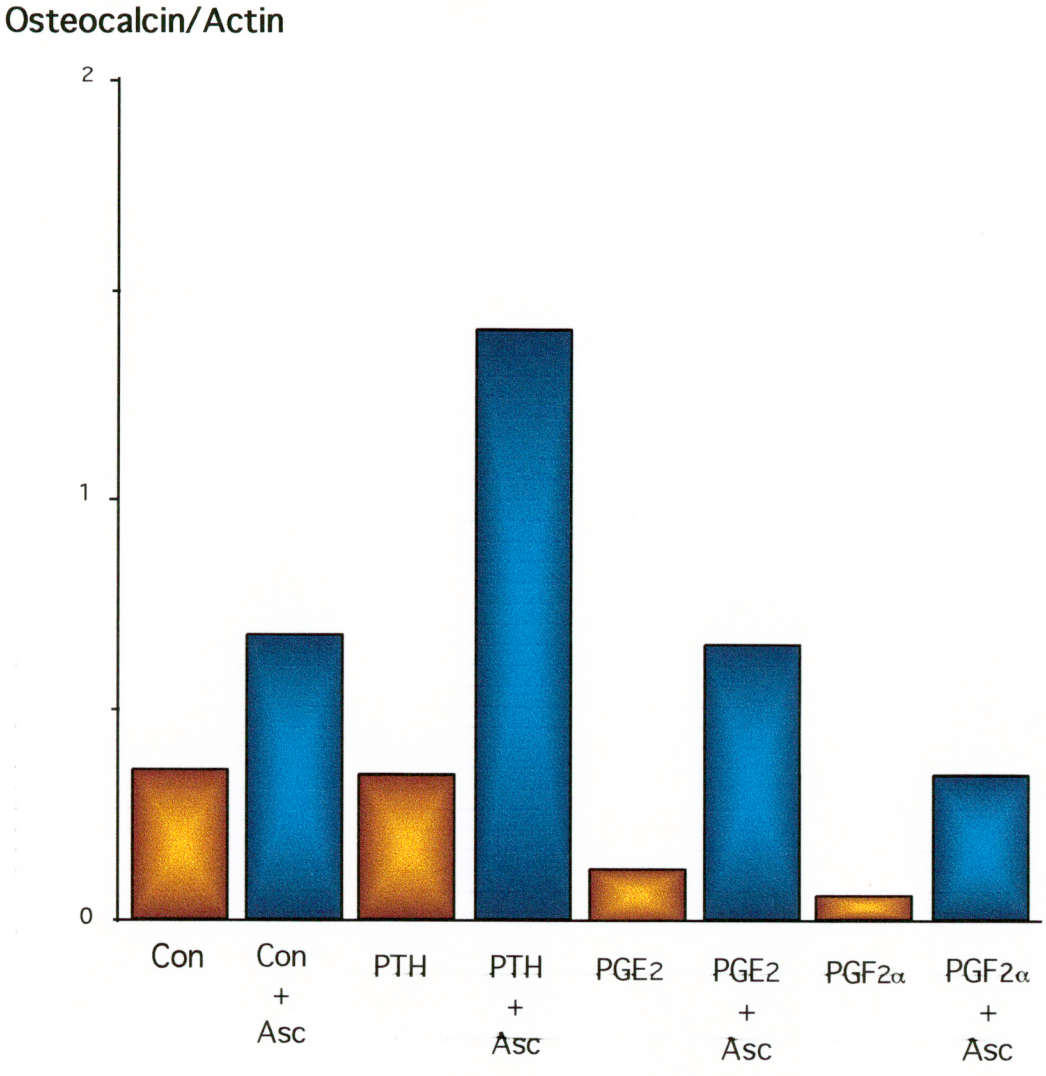
Osteocalcin/Actin
2
1
0
Con
Con + Asc
PTH
PTH + Asc
PGE2
PGE2 + Asc
PGF2α
PGF2α + Asc

Figure 11e

Figure 12a PTH, PGE_2 and $PGF_{2\alpha}$ at 1 mM induce ICER MC3T3-E1 cells cultured for three weeks in the presence or absence of ascorbic acid.

MC3T3-E1 cells were grown in the presence or absence of 50 μg/ml phosphoascorbate for 3 weeks. Cells were treated with 10 nM PTH, 1 μM PGE_2 or $PGF_{2\alpha}$ for 2 h. RNA was extracted and subjected to RT-PCR as described in Materials and Methods.

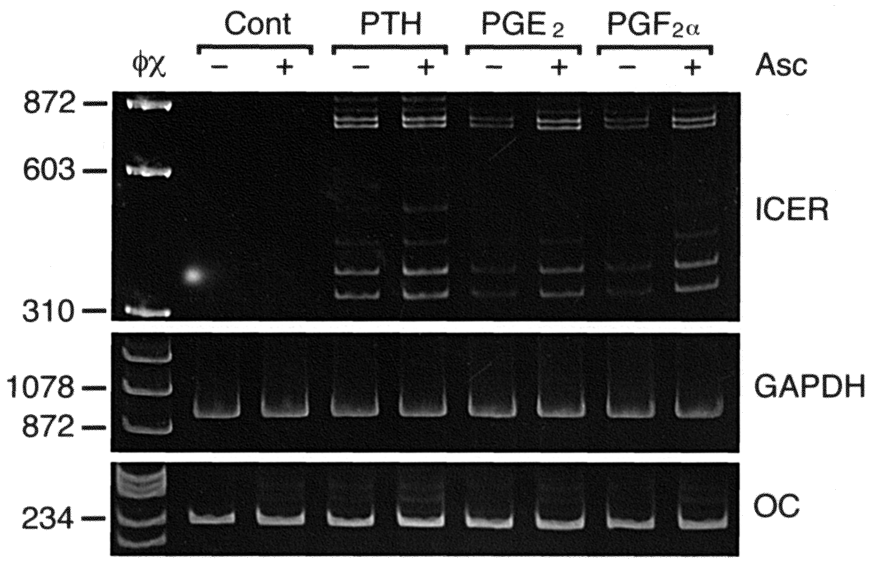
Cont
PTH
PGE2
PGF2α
φχ
– + – + – + – +
Asc
872
603
310
ICER
1078
872
GAPDH
234
OC

Figure 12a

Figure 12b Osteocalcin expression is higher in ascorbate treated compared to ascorbate untreated MC3T3-E1 cells cultured for three weeks.

MC3T3-E1 cells were grown in the presence or absence of 50 μg/ml phosphoascorbate for 3 weeks. Cells were treated with 10 nM PTH, 1 μM PGE_2 or $PGF_{2\alpha}$ for 2 h. RNA was extracted and subjected to Northern blot analysis as described in Materials and Methods.

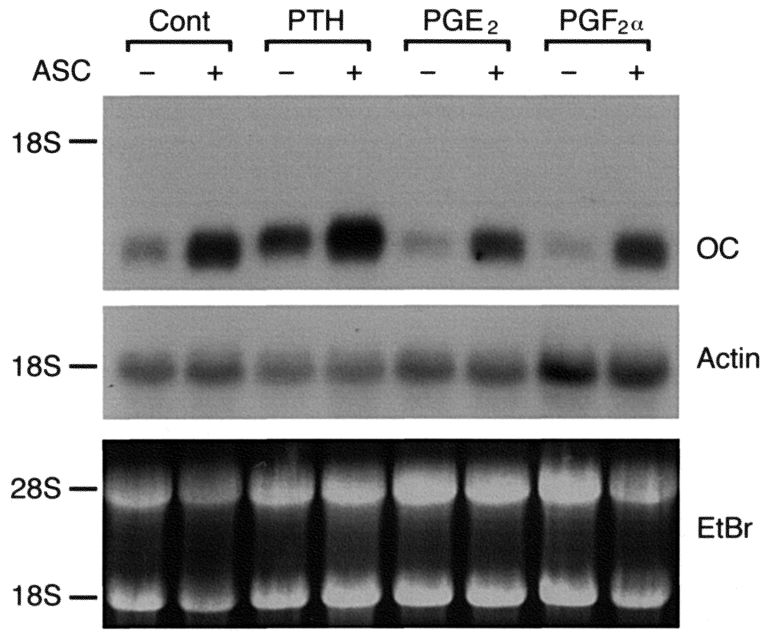

Figure 12b

Figure 12c Densitometry of osteocalcin expression normalized to actin for MC3T3-E1 cells grown in the presence or absence of phosphoascorbate (Asc) for 3 weeks. This graph represents densitometric values for Figure 10b.

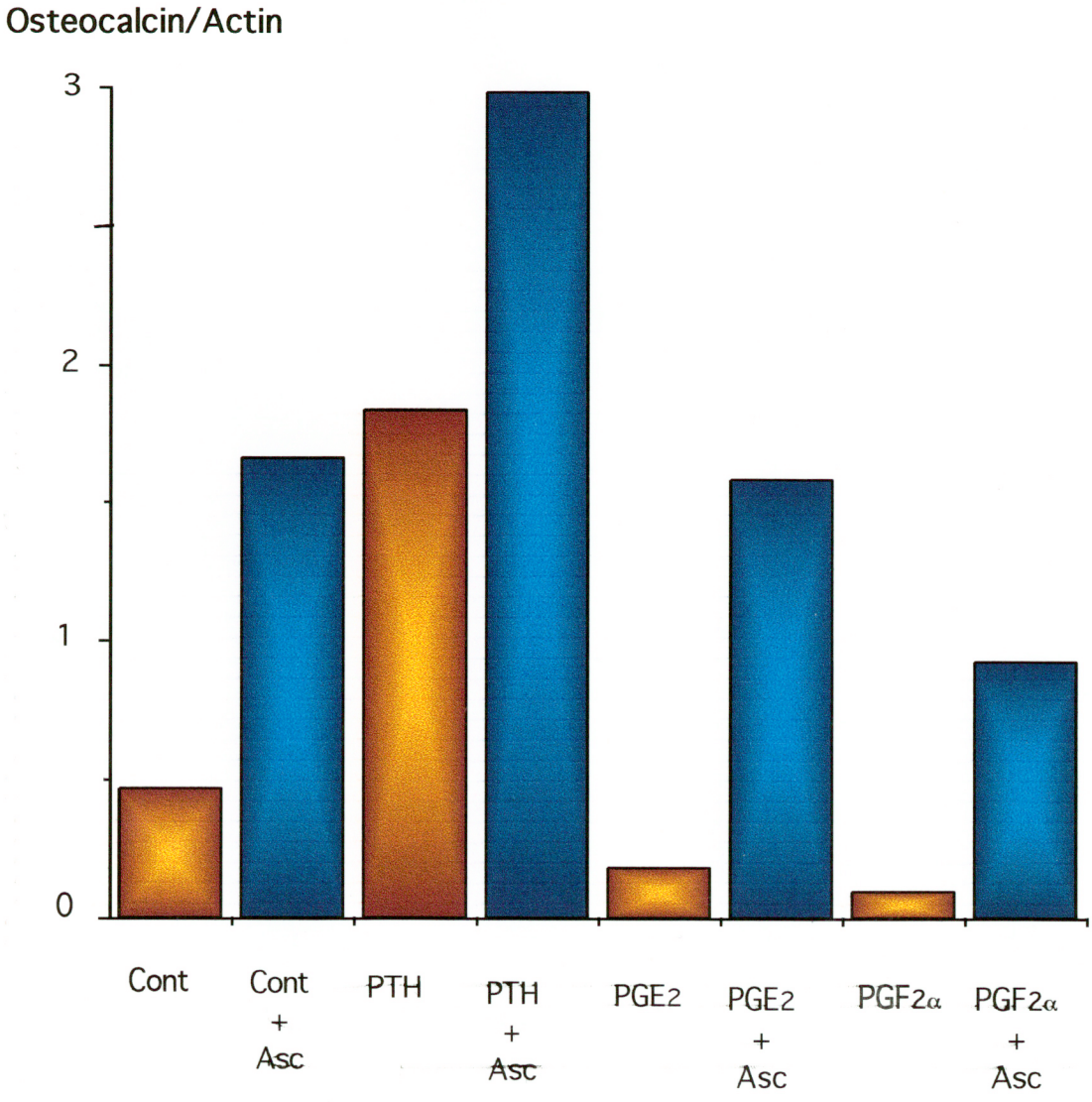
Osteocalcin/Actin
3
2
1
0
Cont
Cont + Asc
PTH
PTH + Asc
PGE2
PGE2 + Asc
PGF2α
PGF2α + Asc

Figure 12e

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