Journal of Cellular Biochemistry 99:411–424 (2006)

ARTICLES

Chronic Hyperglycemia Modulates Osteoblast Gene Expression Through Osmotic and Non-Osmotic Pathways

Sergiu Botolin and Laura R. McCabe*

Molecular Imaging Research Center, 2201 Biomedical Physical Science Building,
Departments of Physiology and Radiology, Michigan State University, East Lansing, Michigan 48824

Abstract Insulin dependent diabetes mellitus (IDDM; type I) is a chronic disease stemming from little or no insulin production and elevated blood glucose levels. IDDM is associated with osteoporosis and increased fracture rates. The mechanisms underlying IDDM associated bone loss are not known. Previously we demonstrated that osteoblasts exhibit a response to acute (1 and 24 h) hyperglycemia and hyperosmolality. Here we examined the influence of chronic hyperglycemia (30 mM) and its associated hyperosmolality on osteoblast phenotype. Our findings demonstrate that osteoblasts respond to chronic hyperglycemia through modulated gene expression. Specifically, chronic hyperglycemia increases alkaline phosphatase activity and expression and decreases osteocalcin, MMP-13, VEGF and GAPDH expression. Of these genes, only MMP-13 mRNA levels exhibit a similar suppression in response to hyperosmotic conditions (mannitol treatment). Acute hyperglycemia for a 48-h period was also capable of inducing alkaline phosphatase and suppressing osteocalcin, MMP-13, VEGF, and GAPDH expression in differentiated osteoblasts. This suggests that acute responses in differentiated cells are maintained chronically. In addition, hyperglycemic and hyperosmotic conditions increased PPARγ2 expression, although this increase reached significance only in 21 days chronic glucose treated cultures. Given that osteocalcin is suppressed and PPARγ2 expression is increased in type I diabetic mouse model bones, these findings suggest that diabetes-associated hyperglycemia may modulate osteoblast gene expression, function and bone formation and thereby contribute to type I diabetic bone loss. J. Cell. Biochem. 99: 411–424, 2006.

Key words: bone; osteoblast differentiation; hyperglycemia; osmotic stress; diabetes; glucose

Insulin dependent diabetes mellitus (IDDM; type I) is a chronic disease stemming from little or no insulin production and elevated blood glucose levels. IDDM is associated with many complications including neuropathy, nephropathy, retinopathy and decreased bone mineral density. While the later complication has not received much attention, it is clear that IDDM is associated with decreased bone mass [Levin et al., 1976; McNair et al., 1981; Wiske et al., 1982; Hui et al., 1985; Buysschaert et al., 1992; Piepkorn et al., 1997; Gunczler et al., 1998; Hampson et al., 1998; Tuominen et al., 1999; Kemink et al., 2000], osteoporosis [Auwerx et al., 1988; Krakauer et al., 1995; Munoz-Torres et al., 1996; Kemink et al., 2000], and increased fracture rates [Bouillon, 1991; Meyer et al., 1993; Forsen et al., 1999; Schwartz et al., 2001]. A recent clinical study found that 67% of male and 57% of female patients with IDDM suffered from osteopenia of the femoral neck and/or lumbar spine [Kemink et al., 2000] and 14%–20% of IDDM patients age 20–56 met the criteria for more extensive bone loss, osteoporosis [Munoz-Torres et al., 1996; Kemink et al., 2000].

Clinical and basic research indicates that modulation of osteoblast rather than osteoclast activity is involved in diabetic bone loss. For example, in the streptozotocin induced mouse model of IDDM, we demonstrated through serum marker analyses and histology that osteoclast number and activity is not influenced by IDDM [Botolin et al., 2005] similar to reports by others [Verhaeghe et al., 1990; Sasaki et al., 1991]. In contrast, osteoblast function/maturation is suppressed and marrow adiposity is

Grant sponsor: NIH (to L.R.M.); Grant number: DK061184.

*Correspondence to: Laura R. McCabe, PhD, Michigan State University, Department of Physiology, 2201 Biomedical Physical Science Bldg., East Lansing, MI 48824.
E-mail: Mccabel@msu.edu

Received 4 October 2005; Accepted 29 December 2005
DOI 10.1002/jcb.20842

increased in IDDM mice. Studies by Bouillon et al. [1995] and others support an osteoblast maturation defect based on serum levels of early osteoblast markers (peptide of procollagen, PICP), which remain normal while later stage markers such as osteocalcin are decreased in diabetes. Similarly, we also found a decrease in serum levels of osteocalcin and osteocalcin mRNA levels in bones of IDDM mice. Our additional finding of increased expression of PPARγ2, aP2 and resistin in streptozotocin-induced diabetic mice corresponded with increased adipocyte maturation and suggested the possibility that IDDM may also affect lineage selection of mesenchymal stem cells, leading to adipocyte rather than osteoblast maturation.

The mechanism accounting for IDDM associated bone loss is unknown. Many studies focus on the anabolic role of insulin and IGF-1 [Hough et al., 1981; Verhaeghe et al., 1992; Haffner and Bauer, 1993; Krakauer et al., 1995; Verhaeghe et al., 1997; McCarthy et al., 2001]. It is clear that insulin is involved in the complications of diabetes based on the finding that insulin treatment can reduce diabetic complications [McNair et al., 1981; Goodman and Hori, 1984; Shyng et al., 2001]. However, whether this is a direct effect or secondary to insulin's restoration of other serum factors is not known. One major consequence of insulin treatment is maintenance of euglycemia, which in itself could be important for avoiding complications [Brownlee et al., 1984]. Reports in other systems and in bone have suggested that elevated glucose can contribute to diabetic complications through a variety of mechanisms including increasing reactive oxygen species [Wolff and Dean, 1987; Hunt et al., 1990], polyol-pathway activity [Gabbay, 1973; Larkins and Dunlop, 1992; Inaba et al., 1997], protein kinase C activity [Craven et al., 1990; Wolf et al., 1991; Lee et al., 1994; Ceolotto et al., 1999], and/or nonenzymatic glycosylation of key proteins such as collagen I or IGF-1 [Brownlee et al., 1984; Bucala et al., 1984; Locatto et al., 1993; Katayama et al., 1996; McCarthy et al., 1997, 2001]. In addition, cells can also respond to hyperglycemia through an osmotic response. Because osteoblasts express glucose transporters, GLUT-1 and -3 [Thomas et al., 1996a,b], with low K_m (1–2 mM and $<$1 mM, respectively), glucose transport is maximal at euglycemic state (glucose concentration of 3–5.5 mM) so an increase in extracellular glucose could be an osmotic stress.

During osmoadaptation to extracellular hyperosmotic conditions, virtually all cells undergo a volume change and shrink [Chamberlin and Strange, 1989; Kwon and Handler, 1995]. Adaptive responses include modulation of membrane ion transporters (short-term) and/or metabolic pathways (long-term) that draw water back into the cell to partially restore cell volume and intracellular solute concentration [Grinstein et al., 1986; Bohren et al., 1989; Kwon et al., 1992; Uchida et al., 1992; O'Donnell, 1993; Parker, 1993; Agre et al., 1995; Kwon and Handler, 1995; O'Donnell et al., 1995; Haussinger, 1996; Lytle and Forbush, 1996; Gatsios et al., 1998; Jenq et al., 1998; Sheikh-Hamad et al., 1998; Kapus et al., 1999; Nadkarni et al., 1999; Roger et al., 1999; Obata et al., 2000; Park et al., 2000; Erickson et al., 2001]. Previously we demonstrated that acute (24 h) hyperglycemia and its associated hyperosmolality are capable of modulating osteoblast signaling pathways, gene expression and phenotype [Zayzafoon et al., 2000, 2002]. Specifically, osteocalcin expression in day 14 osteoblasts was decreased, while expression of collagen I and c-jun was increased.

Given that diabetes is a chronic disease associated with chronic extracellular glucose elevation, we asked the question "Does chronic hyperglycemia and or hyperosmolarity lead to chronic changes in osteoblast gene expression?" We focused on genes that we have demonstrated to be modulated in IDDM mice and we also compared our response to short-term treatments (48 h) to distinguish between acute versus long-term responses, with the thought that perhaps chronic treatment leads to an adaptive response in osteoblasts that results in normalization of gene expression. Our results demonstrate that chronic hyperglycemia significantly influences osteoblast gene expression causing increased alkaline phosphatase expression and decreased osteocalcin, MMP-13, VEGF, and GAPDH expression. Of these genes, only MMP-13 mRNA levels exhibit a similar suppression in response to hyperosmotic conditions (mannitol treatment). For most genes, effects were seen early on (day 5 or 14) and were maintained. Acute hyperglycemia treatment (48 h) caused a similar modulation of gene expression but effects were predominantly seen only in fully differentiated osteoblasts. The

changes in gene expression suggest that later stages of osteoblast differentiation are suppressed by hyperglycemia and suggest a trend toward increased expression of early markers of adipocyte phenotype. In combination with the finding that osteoblast marker genes are suppressed in diabetic mouse bones [Botolin et al., 2005], our in vitro findings suggest that diabetic hyperglycemia may contribute to the suppression of osteoblast differentiation and bone loss in diabetic mice.

MATERIALS AND METHODS

Mouse Plasma Measurements

Diabetes was induced in adult (15-week-old) male Balb/c mice (Harlem Laboratories, Houston, TX) by streptozotocin injection (40 μg/g body weight in 0.1 citrate buffer) for 5 days [Pechhold et al., 2001; Szkudelski, 2001]. Measurements of non-fasting blood glucose levels were made at 7, 14, and 28 days after confirmation of diabetes. Blood was obtained from the lateral saphenous vein of non-fasted mice (controls and diabetics) and glucose measured with a glucometer (Accu-Check instant, Boehringer Mannheim Corporation, Indianapolis, IN). In addition, serum osmolality, defined as the expression of the total number of solute particles dissolved in 1 kg of solvent (International Federation of Clinical Chemistry), was determined using a vapor pressure osmometer (Wescor, Inc., Logan, UT).

Cell Culture System

MC3T3-E1 cells [Sudo et al., 1983], subcloned for maximal alkaline phosphatase staining and mineralization, were used for all studies. Osteoblasts were seeded at 5,000 cells/cm^2 surface area and fed daily with alpha-MEM containing 7% fetal calf serum (a final glucose concentration of 5.5 mM). After 7 days, the media is supplemented with 25 μg/ml ascorbic acid and 2 mM beta-glycerol phosphate (Sigma, St. Louis, MO) to promote osteoblast differentiation and bone formation. Treatment with glucose or mannitol began 24 h after seeding (on day 1) and continued with each feeding until day 7, 14, 21, or 29.

Determination of Alkaline Phosphatase Activity and Mineralization

Alkaline phosphatase staining was performed by incubating cell layers for 30 min at 37°C with 0.5 mg/ml naphthol AS-MX phosphate disodium salt with 1 mg/ml Fast Red TR salt (Sigma Chemical Co.) in a 10.2 M Tris buffer, pH 8.4. Alkaline phosphatase quantitation was performed by solubilizing the precipitated salt in 100% trichloracetic acid and reading at 540 nm as previously described [McCabe et al., 1996]. Mineralization was determined by using a 0.02 M solution of alizarin red in water and incubating at room temperature for 20 min. Wells were rinsed several times to unincorporated dye. Quantitation was performed by extracting the dye from the wells with 10% cetylpyridinum choride solution and reading at 570 nm. Oil Red-O solution was used to stain for lipid and quantitated by adding 100% ethanol to each well and reading at 508 nm.

DNA Analysis

DNA quantification was performed with CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, OR) according to instructions provided by manufacturer. Specifically, frozen cell pellets were thawed to room temperature and lysed in 1 ml of 1X CyQUANT GR working solution and spun for 5 min at 3000 RPM. Samples were analyzed for fluorescence using a fluorescence microplate reader with filters appropriate for 480 nm excitation and 520 nm emission. Values were expressed relative to day 7 control levels.

RNA Analysis

Total RNA was extracted according to the method of Chomczynski and Sacchi as previously described [Chomczynski and Sacchi, 1987; McCabe et al., 1995] and integrity was verified by formaldehyde-agarose gel electrophoresis. Synthesis of cDNA was performed by reverse transcription with 2 μg of total RNA using the Superscript II kit with oligo $dT_{(12-18)}$ primers as described by the manufacturer (Invitrogen, Carlsbad, CA). cDNA (1 μl) was amplified by PCR in a final volume of 25 μl using BioRad SYBER Green Mix (BioRad, Hercules, CA) with 10 pmol of each primer (Integrated DNA Technologies, Coralville, IA). Runx2 was amplified using 5′-GAC AGA AGC TTG ATG ACT CTA AAC C-3′ and 5′-TCT GTA ATC TGA CTC TGT CCT TGT G-3′ [Ontiveros et al., 2004]. Alkaline phosphatase was amplified using 5′-CGTAATCTACC-ATGGAGACATTTTC-3′ and

5′-GACTGTGGTTACTGCTGATCATTC-3′. Collagen I was amplified using 5′-AAG CCT CTT TCT CCT CTC TGA CC-3′. Osteocalcin was amplified using 5′-ACG GTA TCA CTA TTT AGG ACC TGT G-3′ and 5′-ACT TTA TTT TGG AGC TGC TGT GAC-3′ [Ontiveros and McCabe, 2003]. Matrix metalloprotease 13 (MMP-13, collagenase 3) was amplified using 5′-CCA CTG TCC TTG TAG TGG CTG TTA-3′ and 5′-GGA GCC ACA GAT GAG CAC AGA TAA-3′. PPARγ2 was amplified using 5′-TGA AAC TCT GGG AGA TTC TCC TG-3′ and CCA TGG TAA TTT CTT GTG AAG TGC-3′ [Kast-Woelbern et al., 2004]. Genes associated with hypoxic responses were also examined: glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified using 5′-GTG TAC ATG GTT CCA GTA TGA CTC C-3′ and 5′-AGT GAG TTG TCA TAT TTC TCG TGG T-3′ and vascular endothelial growth factor (VEGF) was amplified using 5′-ATA TCA GGC TTT CTG GAT TAA GGA C-3′ and 5′-CAG ACG AAA GAA AGA CAG AAC AAA G-3′. Cyclophilin was not modulated by sugar treatments and was used as an internal PCR control [Lewis and Hughes-Fulford, 2000; Trogan et al., 2002] (primers: 5′-ATT CAT GTG CCA GGG TGG TGA C-3′ and 5′-CCG TTT GTG TTT GGT CCA GCA-3′). Specifically, levels over the time course and between conditions ranged between 0.97 and 1, relative to day 7 controls set at 1. Real time PCR was carried out for 40 cycles using the iCycler (Bio-Rad, Hercules, CA) and data were evaluated using the iCycler software. RNA-free samples, a negative control, did not produce amplicons. Melting curve and gel analyses (sizing, isolation, and sequencing) were used to verify single products of the appropriate base pair size.

Statistical Analysis

All statistical analyses were performed using Microsoft excel data analysis program for *t*-test analysis. Experiments were repeated at least three times. Values are expressed as a mean ± SE.

RESULTS

Prior to beginning our in vitro studies we directly measured blood glucose levels and plasma osmolality in normal and diabetic Balb/c mice. As expected, diabetic animals exhibited elevated blood glucose levels of 300–500 mg/dl [Botolin et al., 2005]. In terms of molar quantities, non-fasting control mice had glucose levels of 9.7 ± 0.2 mM (levels higher than standard alpha-MEM cell culture medium, 5.5 mM) while diabetic mice had glucose levels of 39.2 ± 3.5 mM; this corresponds to an average increase of 30 mM glucose. Similarly, diabetic mice exhibit an elevation in blood osmolality (335.8 ± 3.3 mmol/kg) compared to controls (305.2 ± 2.2 mmol/kg); this represents an increase of 30 ± 5 mmol/kg [Botolin et al., 2005]. Based on these findings, osteoblasts were treated in vitro with 30 mM glucose to attain a final concentration of 35.5 mM glucose and a final osmolality of 322 mmol/kg (compared to 295 in control cultures). To control for the effects of glucose-associated hyperosmolarity, osteoblasts were treated with 30 mM mannitol to attain a final media concentration of glucose equal to control cultures (5.5 mM glucose) but with an osmolarity of 322 mOsm.

First, we examined if chronic glucose treatment has a long-term influence on cell number, since changes in cell number could ultimately affect osteoblast phenotype and gene expression. DNA levels per tissue culture plate were determined at 7, 14, 21, or 29 days after plating. Total DNA levels (an indicator of cell number) did not significantly differ between control, glucose and mannitol treated cultures at day 7 (1.0 ± 0.2, 1.1 ± 0.1, 1.4 ± 0.1, relative to day 7 controls, respectively) but by day 14 (1.9 ± 0.3, 1.7 ± 0.2, 1.7 ± 0.2 relative to day 7 controls, respectively) and 21–28 days (2.7 ± 0.2, 2.2 ± 0.2, 2.2 ± 0.1) a gradual decrease in cell number was noted in glucose and mannitol treated cultures. This decrease did not reach statistical significance. Visual examination of the cells under bright field did not demonstrate any remarkable differences in osteoblast morphology or confluency under hyperglycemic and/or hyperosmotic conditions (Fig. 1). However, examination of histological staining demonstrated that alkaline phosphatase activity was increased in glucose treated cultures (Fig. 1). Staining for mineralization did not appear to differ between conditions (data not shown).

Next, analyses of gene expression were carried out. Four subsets of genes were examined and included those associated with early osteoblast maturation, late osteoblast maturation, adipocyte maturation, and metabolic stress/hypoxia. For each set of genes, chronic and 48 hour treatments were examined. Cyclophilin mRNA levels were not significantly modulated

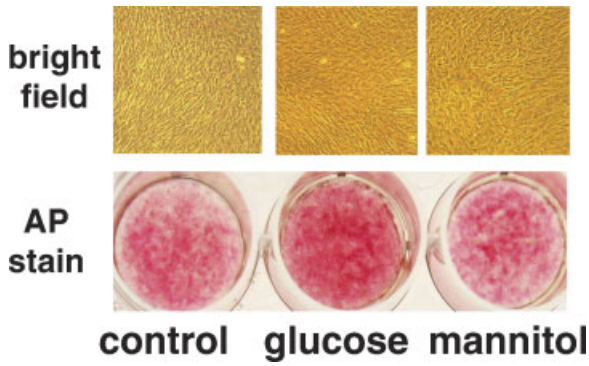

Fig. 1. Osteoblast morphology and number does not change under chronic hyperglycemia, but alkaline phosphatase activity is increased. Mouse osteoblasts (MC3T3-E1 cells) were treated with 30 mM glucose or mannitol or remained untreated (control). Sugars were added with each feeding. Shown is a brightfield image of confluent cultures taken at day 14 and a representative photo of alkaline phosphatase activity staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

by glucose or mannitol treatments and were used as a housekeeping control gene. Figure 2 depicts the time course of early osteoblast maturation markers (runx2, alkaline phosphatase, and collagen I mRNA levels) under control, chronic glucose (30 mM) or chronic mannitol (30 mM) treatments. While runx 2 mRNA levels were elevated at 7, 14, and 29 days under hyperglycemic conditions, the change was not significant compared to untreated osteoblasts. However, the marked induction of alkaline phosphatase expression by hyperglycemia was significant at all time points and was consistent with the observed elevation in alkaline phosphatase staining (Fig. 1). This effect was not osmotic in nature since chronic treatment with mannitol did not affect alkaline phosphatase expression. Expression of collagen I was not markedly modulated by any treatment, but at day 29 glucose levels reached statistical significance in glucose compared to control cultures. To determine if these changes with long-term treatment could be contributed to a short-term response to the continual glucose addition, we examined if alkaline phosphatase or collagen I mRNA levels were altered by 48 hours of glucose or mannitol treatment. The short-term responses were variable and did not exactly follow long-term responses (Fig. 3). Again, collagen I expression was not markedly modulated by any treatment, but its levels were modestly but significantly affected in day 7 mannitol treated cells. Alkaline phosphatase expression was decreased by short-term mannitol

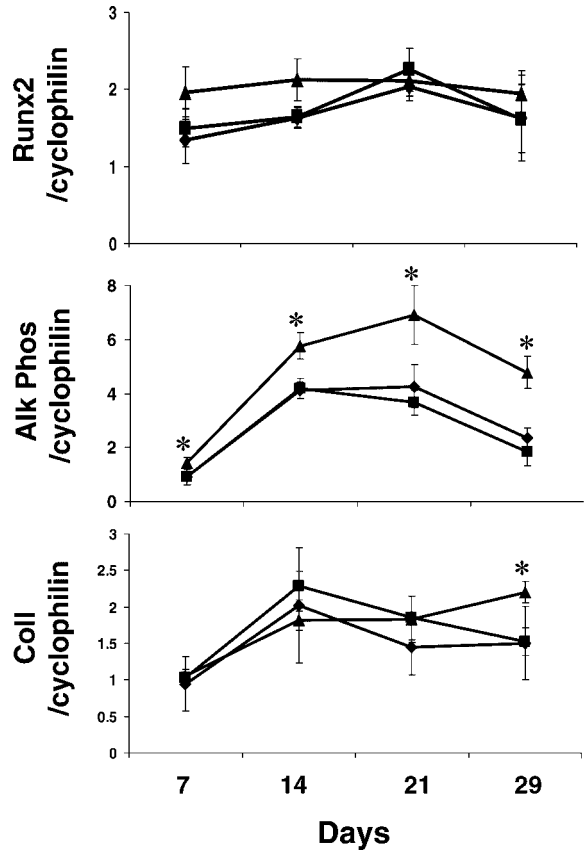

Fig. 2. Expression of early and mid stage markers of osteoblast differentiation is modulated by chronic hyperglycemia. Osteoblasts were chronically treated with 30 mM glucose (triangles) or mannitol (diamonds) or were maintained under standard 5.5 mM glucose conditions (control, squares). Cells were harvested at 7, 14, 21, or 29 days and expression of runx2, alkaline phosphatase and collagen I was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $^*P < 0.05$.

treatment at 14 days (unlike any response seen in chronic treatment), but tended to increase under short-term hyperglycemic conditions at 21 days and was significantly increased at 29 days (Fig. 3).

As expected, examination of late stage markers of osteoblast differentiation, osteocalcin, and collagenase 3/MMP-13 [Stein et al., 1996; Winchester et al., 2000], demonstrated an increase in expression in control cells with increasing time in culture (Fig. 4, control). However under chronic hyperglycemic conditions osteocalcin and MMP-13 mRNA levels were decreased by hyperglycemia; this is in contrast to hyperglycemia-induced elevation of early stage osteoblast maturation (Fig. 2). Specifically, at 14 and 21 days post-seeding osteocalcin expression was decreased in glucose treated cultures and by

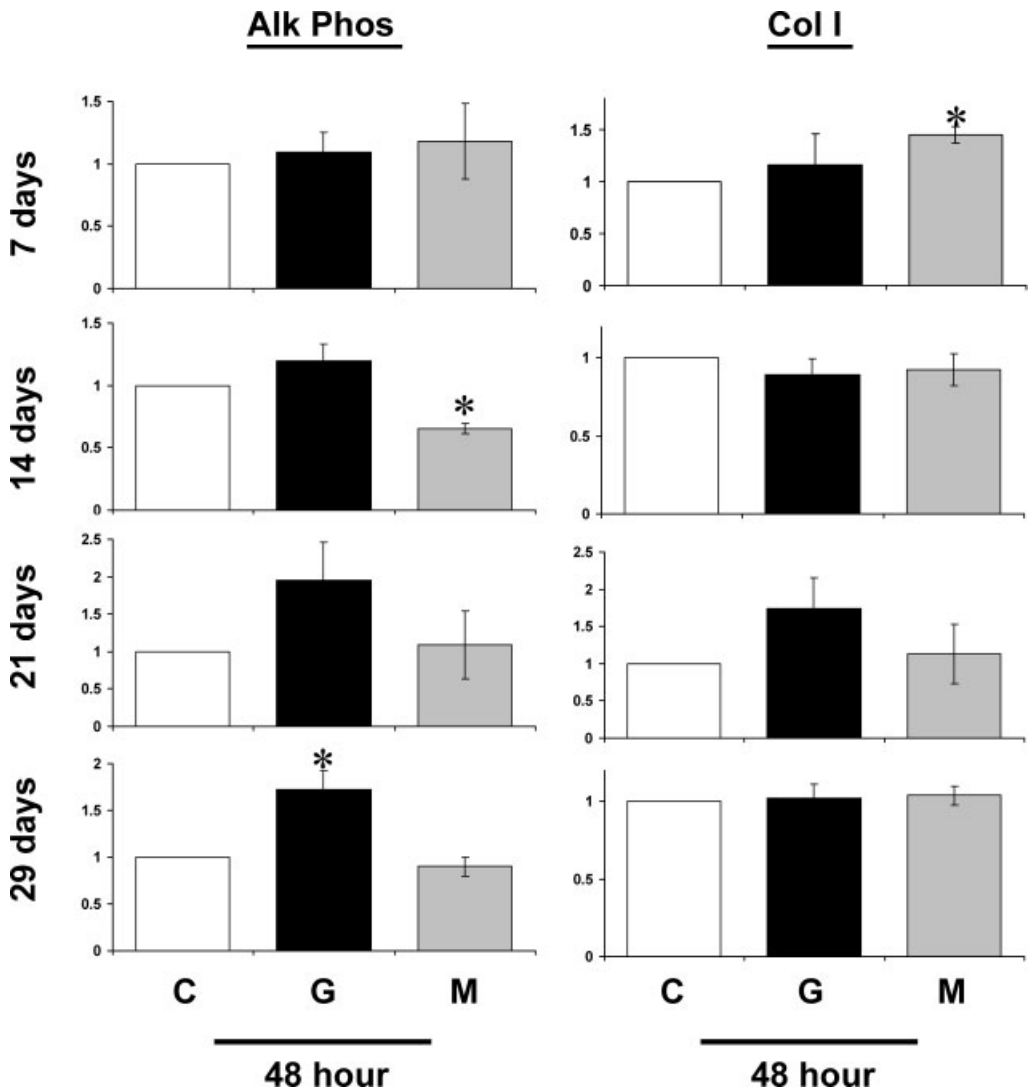

Fig. 3. Expression of early and mid stage markers of osteoblast differentiation is modulated by 48-h hyperglycemic conditions. Osteoblasts were treated for 48 h with 30 mM glucose (black bars) or mannitol (gray bars) or were maintained under standard 5.5 mM glucose conditions (control, white bars). Cells were harvested at 7, 14, 21, or 29 days and expression of alkaline phosphatase and collagen I was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $^*P < 0.05$.

29 days the decrease was statistically significant (Fig. 4). Mannitol treated cells also showed a trend toward decreased osteocalcin expression at days 14 and 21, however the difference was never statistically significant. In contrast, MMP-13 expression was significantly suppressed by both glucose and mannitol treatments at 7, 14, 21, and 29 days, suggesting that chronic hyperglycemia-associated hyperosmotic stress is responsible for the suppression in MMP-13 expression in osteoblasts. When short-term (48 h) responses were examined (Fig. 5), both osteocalcin and MMP-13 mRNA levels were significantly suppressed by glucose treatment in day 21 and 29 cells. This reflects the suppression seen with chronic treatment. While acute mannitol treatment did not affect MMP-13 mRNA levels in maturing osteobalsts (Fig. 5), it did increase MMP-13 expression in day 7 cells (similar to Col I regulation). Acute mannitol treatment also suppressed osteocalcin mRNA levels in day 29 osteobalsts (Fig. 5).

Previously we have shown that diabetes type I is associated with increased bone PPARγ2

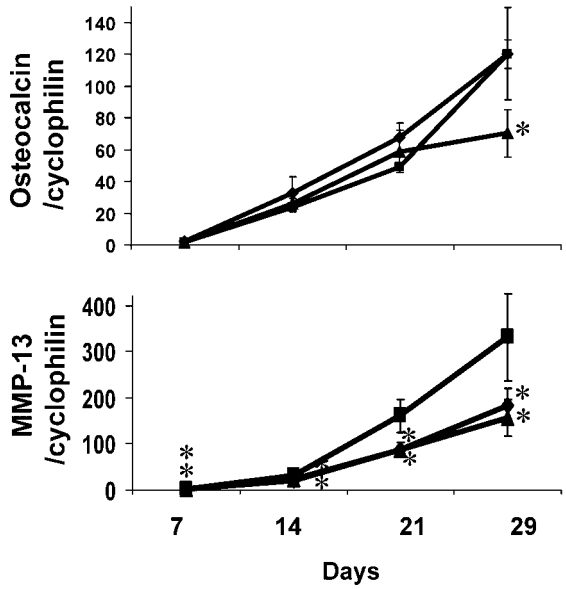

Fig. 4. Expression of late stage markers of osteoblast differentiation is modulated by chronic hyperglycemia and, in the case of MMP-13, hyperosmolarity. Osteoblasts were chronically treated with 30 mM glucose (triangles) or mannitol (diamonds) or were maintained under standard 5.5 mM glucose conditions (control, squares). Cells were harvested at 7, 14, 21, or 29 days and expression of osteocalcin and MMP-13 was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $*P < 0.05$.

mRNA levels and marrow adiposity [Botolin et al., 2005]. Therefore, we next asked the question: can chronic hyperglycemia or hyperosmotic stress directly influence PPARγ2 mRNA expression, a marker of early stage adipocyte differentiation? Figure 6 demonstrates that hyperglycemia and its associated hyperosmotic stress can increase mRNA levels of PPARγ2. Specifically, chronic glucose treatment and mannitol treatment for 14 and 21 days lead to nearly a two-fold induction in PPARγ2 mRNA levels. However, the response was variable and reached statistical significance only in chronically glucose treated day 21 cultures; lipid staining at this time point did not differ between conditions (data not shown). The PPARγ2 response to 48 hour glucose or mannitol treatment did not demonstrate a significant difference between conditions (data not shown).

Given that diabetes type I is associated with decreased circulation, we next examined the expression of genes associated with vascularization and hypoxic /glycolytic responses: vascular endothelial growth factor (VEGF) and glyceraldehyde phosphate dehydrogenase (GAPDH). Figure 7 demonstrates that basal expression of VEGF and GAPDH is increased with osteoblast differentiation and that chronic hyperglycemia is effective at suppressing the expression of both of these genes at days 21 and 29. Chronic hyperosmolarity did not cause a significant suppression, but did trend toward decreasing VEGF and GAPDH and gave an intermediate response between control and glucose treated cells. Acute hyperglycemic treatment at day 29 was also effective at suppressing both VEGF and GAPDH mRNA levels (Fig. 8).

DISCUSSION

Elevated blood glucose level is a key characteristic of type I diabetes. Chronic hyperglycemia itself is thought to contribute to diabetic complications. To examine the influence of chronic hyperglycemia and its associated hyperosmotic stress on osteoblast phenotype, mouse osteoblasts were cultured in vitro under pathologic conditions of hyperglycemia as seen in streptozotocin-induced type I diabetic mice. In addition, cells were exposed to pathologic hyperosmolality at levels also seen in diabetic mice. This approach allows the distinction between responses directly related to hyperglycemia and hyperosmolality versus those that are secondary to other diabetes-modulated factors (serum hormones and nutrients) or involve other cell types (immune, hematopoetic, neural, osteocyte, etc....). Our findings demonstrate that indeed osteoblasts respond to chronic hyperglycemia through modulated gene expression. Specifically, chronic hyperglycemia increases alkaline phosphatase activity and expression and decreases osteocalcin, MMP-13, VEGF, and GAPDH expression. Changes were apparent by day 7 for alkaline phosphatase and MMP-13 and were maintained throughout the rest of the time course. Of these genes, only MMP-13 mRNA levels exhibit a similar suppression in response to hyperosmolarity (mannitol treatment). Examination of 48-hour treatment indicates that induction of alkaline phosphatase and suppression of osteocalcin, MMP-13, VEGF, and GAPDH can be mediated acutely in day 29 osteoblasts (and for osteocalcin and MMP-13, also in day 21 osteoblasts). In addition, similar to in vivo gene analyses [Botolin et al., 2005], osteoblasts exhibit a trend toward increased PPARγ2

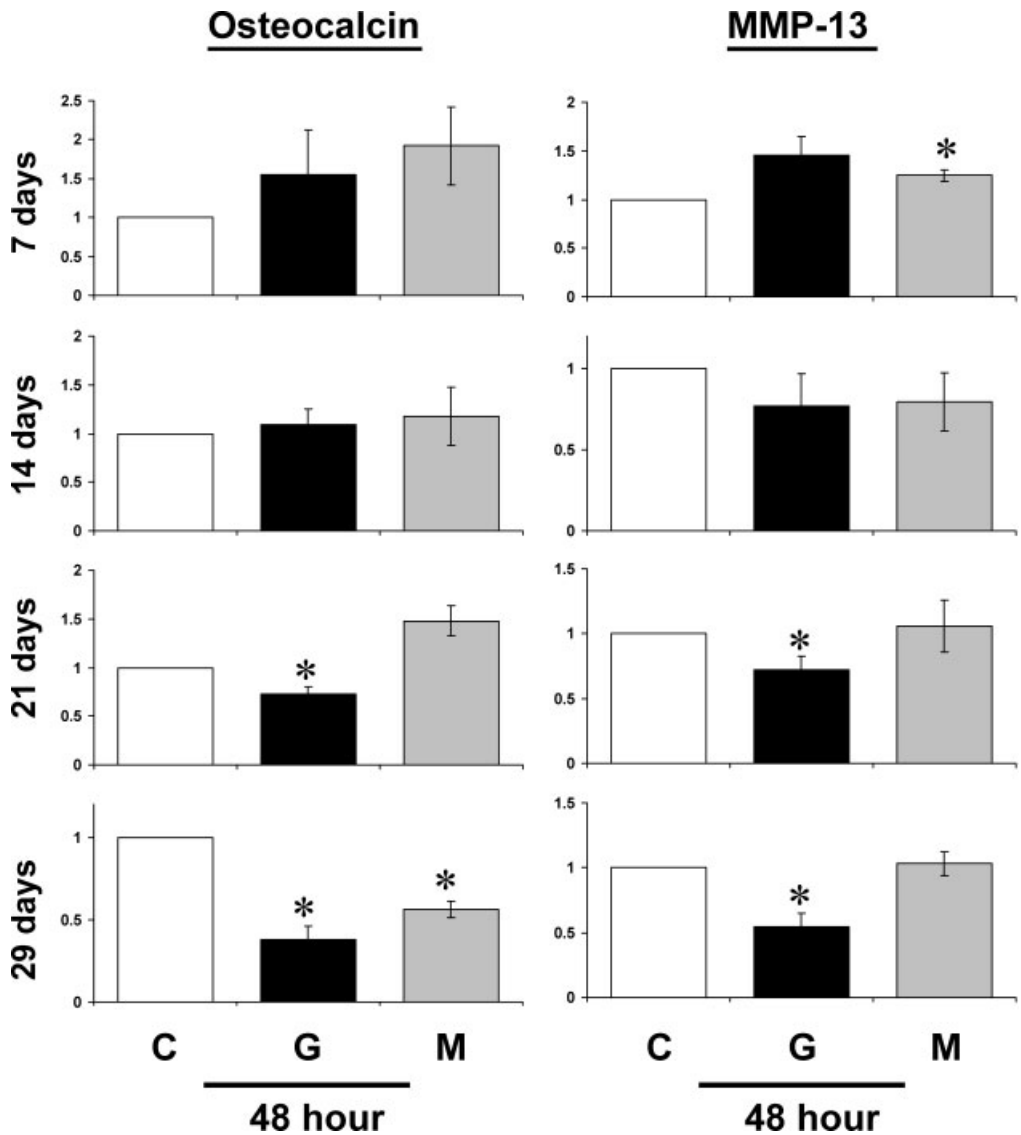

Fig. 5. Expression of late stage markers of osteoblast differentiation is modulated by 48-h hyperglycemic conditions. Osteoblasts were treated for 48 h with 30 mM glucose (black bars) or mannitol (gray bars) or were maintained under standard 5.5 mM glucose conditions (control, white bars). Cells were harvested at 7, 14, 21, or 29 days and expression of osteocalcin and MMP-13 was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $^*P < 0.05$.

expression, suggesting a potential contribution by hyperglycemic and hyperosmotic conditions toward this phenomenon.

Interestingly, chronic treatment tended to influence osteoblast gene expression at significantly earlier time points compared to acute treatments. For example, acute hyperglycemia suppresses osteocalcin and MMP-13 in day 21 and 29 cells, but not in day 7 or 14 cells (as seen for MMP-13 expression in chronically treated osteoblasts). Alkaline phosphatase, GAPDH and VEGF changes are only seen with acute treatment of day 29 cells, despite chronic treatment causing a significant change day 7, 14, 21 osteoblasts, respectively. These findings suggest that chronic elevation of blood glucose levels in vivo could impact osteoblasts at all stages of maturation, whereas periodic bouts of hyperglycemia will predominantly influence mature osteoblast function.

Similar to our previous studies examining 1 and 24 hour glucose treatments [Zayzafoon

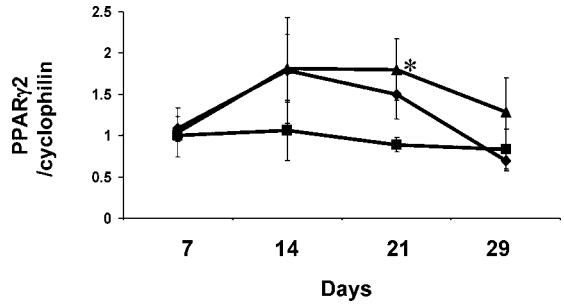

Fig. 6. Expression of adipocyte-phenotype marker, PPARγ2, is increased under chronic hyperglycemic conditions. Osteoblasts were chronically treated with 30 mM glucose (triangles) or mannitol (diamonds) or were maintained under standard 5.5 mM glucose conditions (control, squares). Cells were harvested at 7, 14, 21, or 29 days and PPARγ2 expression was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $*P < 0.05$.

et al., 2000, 2002], we also found that longer incubations (48 hour and chronic 29 day treatments) suppress osteocalcin mRNA levels. Unlike our previous studies, long-term suppression of osteocalcin expression was not hyperosmolality dependent. In fact, modulation of alkaline phosphatase, VEGF, and GAPDH mRNA levels by hyperglycemia was also not

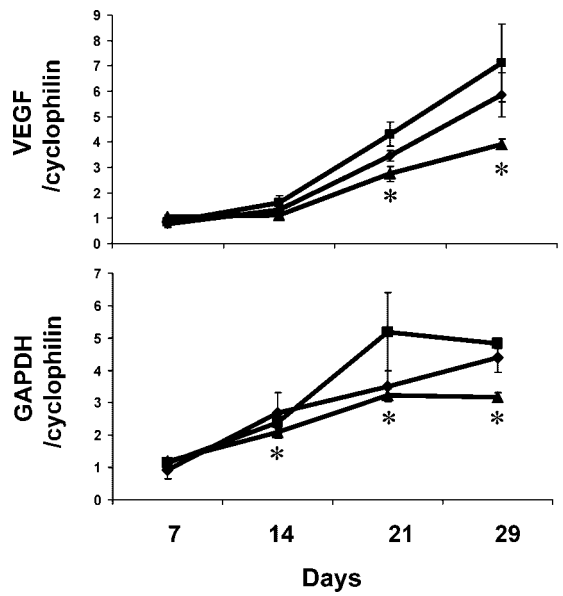

Fig. 7. Expression of VEGF and GAPDH is modulated by chronic hyperglycemia. Osteoblasts were chronically treated with 30 mM glucose (triangles) or mannitol (diamonds) or were maintained under standard 5.5 mM glucose conditions (control, squares). Cells were harvested at 7, 14, 21, or 29 days and expression of VEGF and GAPDH was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $*P < 0.05$.

associated with a hyperosmolality. This suggests that osteoblasts exhibit at least two hyperglycemia responses: an early acute response that is hyperosmolality driven and a late acute and chronic response that is predominantly hyperglycemia driven. These findings are consistent with those of Balint et al. [2001] who reported that chronic hyperglycemia but not hyperosmolality influences calcium uptake in osteoblast cultures in vivo.

Mechanisms accounting for long-term responses to hyperglycemia include activation of the PKC signaling pathway, nonenzymatic glycosylation, modulation of redox state, increased polyol pathway activity, and increased glucose metabolism [Gabbay, 1973; Brownlee et al., 1984; Bucala et al., 1984; Craven et al., 1990; Wolf et al., 1991; Larkins and Dunlop, 1992; Locatto et al., 1993; Lee et al., 1994; Katayama et al., 1996; Inaba et al., 1997; McCarthy et al., 1997; Ceolotto et al., 1999]. The later possibility is of interest since it is related to an observed decrease in medium pH (ranging from 7.4 to 7.1), 48 h after hyperglycemic treatment. The drop did not occur in mannitol treated cultures, which retained a pH of 7.4 hours throughout the experiment. An increase in glucose metabolism would contribute to the suppression of pH by increasing extracellular lactic acid production or another byproduct of glucose metabolism. Previous studies have demonstrated that osteoblasts express acid-sensitive channels [Jahr et al., 2005; Pi and Quarles, 2005] and respond to decreased extracellular pH by decreasing mineralization and gene expression [Brandao-Burch et al., 2005]. However, acid pH is shown to suppress alkaline phosphatase mRNA levels while we see alkaline phosphatase mRNA levels increase in glucose treated cells. This suggests that extracellular pH changes may contribute to some but not all of the changes that we see in osteoblast gene expression under high glucose conditions.

Reduced hypoxia inducible factor (HIF) activity may also contribute to suppression of VEGF and GAPDH by chronic elevated glucose conditions. Hypoxia and its corresponding induction of HIFs are known to upregulate VEGF and GAPDH [Forsythe et al., 1996; Steinbrech et al., 1999; Semenza, 2000; Ontiveros et al., 2004]. Recent studies suggest that hyperglycemia and hyperosmolality can reduce HIF transcriptional activity and hypoxia responsiveness in primary

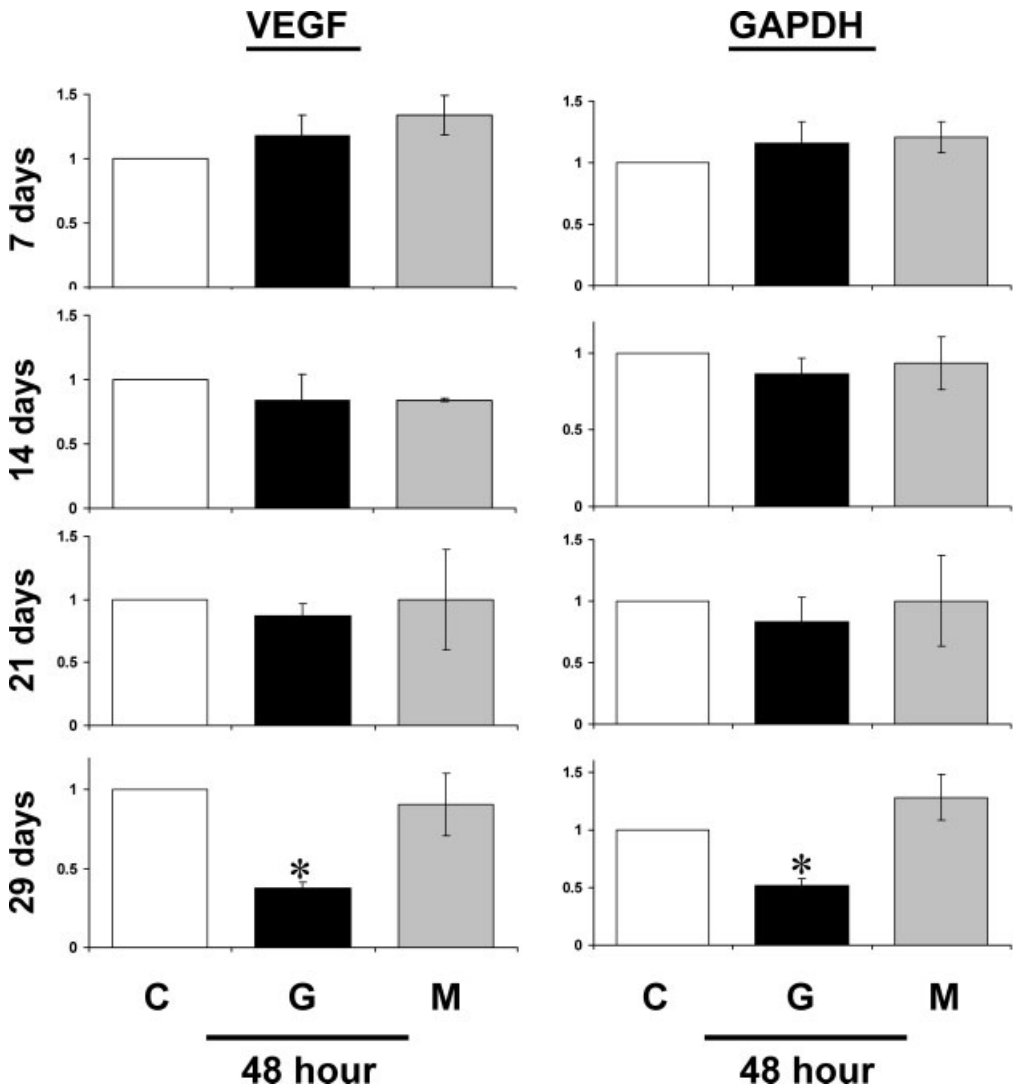

Fig. 8. Expression of late VEGF and GAPDH in differentiated osteoblasts is reduced under 48-h hyperglycemic conditions. Osteoblasts were treated for 48 h with 30 mM glucose (black bars) or mannitol (gray bars) or were maintained under standard 5.5 mM glucose conditions (control, white bars). Cells were harvested at 7, 14, 21, or 29 days and expression of VEGF and GAPDH was determined and expressed relative to cyclophilin (housekeeping gene) levels. Each point represents an average of three separate experiments ± SE, $^*P < 0.05$.

dermal fibroblasts and endothelial cells [Catrina et al., 2004]. A similar response may be occurring in osteoblasts and is in the process of being tested.

While elevated extracellular glucose plays a key role in osteoblast gene regulation, MMP-13 also responds significantly to elevated extracellular mannitol (hyperosmolality) at all time points studied (days 7, 14, 21, 29). This suggests that under chronic hyperglycemia conditions osteoblasts are actively mediating a hyperosmotic response that modulates expression of a subset of genes. Other cells, such as mesangial and endothelial, also mount an osmotic response to pathologic elevations (22 mM) in extracellular glucose [Kreisberg et al., 1994]. In addition, human retinal corneal epithelial cells exhibit changes in MMP-13 (as well as other MMPs) expression in response to hyperosmolar conditions [Li et al., 2004]. However the retinal osmotic-response causes an induction of MMP-13 whereas we see a suppression of MMP-13. These differences may stem from differences in specific transcription factors present in the two

different cell types, retinal epithelial versus osteoblast. For example runx2 is expressed in osteoblasts and binds the MMP-13 promoter (along with ubiquitous AP-1 members) to regulate its transcription [Selvamurugan et al., 1998].

Taken together, we hypothesize that osteoblasts exhibit immediate/early acute changes in gene expression in response to hyperglycemia that are predominantly driven by hyperosmolality. During this period osteoblasts undergo immediate volumetric changes (cell shrinking) induced by hyperglycemia-associated hyperosmolality. With longer incubations, osteoblasts develop adaptive measures to compensate for the hyperosmotic stress, which no longer influence expression of osteoblast marker genes, except for a subset that includes MMP-13. Under these conditions, elevated levels of glucose and its consequences (other than hyperosmolality) are responsible for changes in osteoblast gene expression. The changes that we observed, suppression of markers of late stage osteoblast differentiation, in combination with the finding that osteoblast marker genes are suppressed in diabetic mouse bones [Botolin et al., 2005], suggest that diabetic hyperglycemia may contribute to the suppression of osteoblast differentiation and bone loss in diabetic mice.

ACKNOWLEDGMENTS

We thank Regina Irwin for her technical assistance and insightful suggestions and Gavin Gibson for his helpful comments. This work was funded by a grant from NIH (DK061184) to LRM.

REFERENCES

Agre P, Brown D, Nielsen S. 1995. Aquaporin water channels: Unanswered questions and unresolved controversies. Curr Opin Cell Biol 7:472–483.

Auwerx J, Dequeker J, Bouillon R, Geusens P, Nijs J. 1988. Mineral metabolism and bone mass at peripheral and axial skeleton in diabetes mellitus. Diabetes 37:8–12.

Balint E, Szabo P, Marshall CF, Sprague SM. 2001. Glucose-induced inhibition of in vitro bone mineralization. Bone 28:21–28.

Bohren KM, Bullock B, Wermuth B, Gabbay KH. 1989. The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. J Biol Chem 264:9547–9551.

Botolin S, Faugere MC, Malluche H, Orth M, Meyer R, McCabe LR. 2005. Increased bone adiposity and peroxisomal proliferator-activated receptor-gamma2 expression in type I diabetic mice. Endocrinology 146:3622–3631.

Bouillon R. 1991. Diabetic bone disease [editorial]. Calcif Tissue Int 49:155–160.

Bouillon R, Bex M, Van Herck E, Laureys J, Dooms L, Lesaffre E, Ravussin E. 1995. Influence of age, sex, and insulin on osteoblast function: Osteoblast dysfunction in diabetes mellitus. J Clin Endocrinol Metab 80:1194–1202.

Brandao-Burch A, Utting JC, Orriss IR, Arnett TR. 2005. Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tissue Int 77:167–174.

Brownlee M, Vlassara H, Cerami A. 1984. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann Intern Med 101:527–537.

Bucala R, Model P, Cerami A. 1984. Modification of DNA by reducing sugars: A possible mechanism for nucleic acid aging and age-related dysfunction in gene expression. Proc Natl Acad Sci USA 81:105–109.

Buysschaert M, Cauwe F, Jamart J, Brichant C, De Coster P, Magnan A, Donckier J. 1992. Proximal femur density in type 1 and 2 diabetic patients. Diabete Metab 18: 32–37.

Catrina SB, Okamoto K, Pereira T, Brismar K, Poellinger L. 2004. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. Diabetes 53:3226–3232.

Ceolotto G, Gallo A, Miola M, Sartori M, Trevisan R, Del Prato S, Semplicini A, Avogaro A. 1999. Protein kinase C activity is acutely regulated by plasma glucose concentration in human monocytes in vivo. Diabetes 48:1316–1322.

Chamberlin ME, Strange K. 1989. Anisosmotic cell volume regulation: A comparative view. Am J Physiol 257:C159–C173.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Craven PA, Davidson CM, DeRubertis FR. 1990. Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. Diabetes 39:667–674.

Erickson GR, Alexopoulos LG, Guilak F. 2001. Hyperosmotic stress induces volume change and calcium transients in chondrocytes by transmembrane, phospholipid, and G-protein pathways. J Biomech 34:1527–1535.

Forsen L, Meyer HE, Midthjell K, Edna TH. 1999. Diabetes mellitus and the incidence of hip fracture: Results from the Nord-Trondelag Health Survey. Diabetologia 42: 920–925.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604–4613.

Gabbay KH. 1973. The sorbitol pathway and the complications of diabetes. N Engl J Med 288:831–836.

Gatsios P, Terstegen L, Schliess F, Haussinger D, Kerr IM, Heinrich PC, Graeve L. 1998. Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. J Biol Chem 273:22962–22968.

Goodman WG, Hori MT. 1984. Diminished bone formation in experimental diabetes. Relationship to osteoid maturation and mineralization. Diabetes 33:825–831.

Grinstein S, Furuya W, Cragoe EJ, Jr. 1986. Volume changes in activated human neutrophils: The role of Na^+/H^+ exchange. J Cell Physiol 128:33–40.

Gunczler P, Lanes R, Paz-Martinez V, Martins R, Esaa S, Colmenares V, Weisinger JR. 1998. Decreased lumbar spine bone mass and low bone turnover in children and adolescents with insulin dependent diabetes mellitus followed longitudinally. J Pediatr Endocrinol Metab 11: 413–419.

Haffner SM, Bauer RL. 1993. The association of obesity and glucose and insulin concentrations with bone density in premenopausal and postmenopausal women. Metabolism 42:735–738.

Hampson G, Evans C, Petitt RJ, Evans WD, Woodhead SJ, Peters JR, Ralston SH. 1998. Bone mineral density, collagen type 1 alpha 1 genotypes and bone turnover in premenopausal women with diabetes mellitus. Diabetologia 41:1314–1320.

Haussinger D. 1996. The role of cellular hydration in the regulation of cell function. Biochem J 313:697–710.

Hough S, Avioli LV, Bergfeld MA, Fallon MD, Slatopolsky E, Teitelbaum SL. 1981. Correction of abnormal bone and mineral metabolism in chronic streptozotocin-induced diabetes mellitus in the rat by insulin therapy. Endocrinology 108:2228–2234.

Hui SL, Epstein S, Johnston CC, Jr. 1985. A prospective study of bone mass in patients with type I diabetes. J Clin Endocrinol Metab 60:74–80.

Hunt JV, Smith CC, Wolff SP. 1990. Autoxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. Diabetes 39: 1420–1424.

Inaba M, Nishizawa Y, Shioi A, Morii H. 1997. Importance of sustained high glucose condition in the development of diabetic osteopenia: Possible involvement of the polyol pathway. Osteoporos Int 7:S209–S212.

Jahr H, van Driel M, van Osch GJ, Weinans H, van Leeuwen JP. 2005. Identification of acid-sensing ion channels in bone. Biochem Biophys Res Commun 337: 349–354.

Jenq W, Mathieson IM, Ihara W, Ramirez G. 1998. Aquaporin-1: An osmoinducible water channel in cultured mIMCD-3 cells. Biochem Biophys Res Commun 245:804–809.

Kapus A, Szaszi K, Sun J, Rizoli S, Rotstein OD. 1999. Cell shrinkage regulates Src kinases and induces tyrosine phosphorylation of cortactin, independent of the osmotic regulation of Na^+/H^+ exchangers. J Biol Chem 274: 8093–8102.

Kast-Woelbern HR, Dana SL, Cesario RM, Sun L, de Grandpre LY, Brooks ME, Osburn DL, Reifel-Miller A, Klausing K, Leibowitz MD. 2004. Rosiglitazone induction of Insig-1 in white adipose tissue reveals a novel interplay of peroxisome proliferator-activated receptor gamma and sterol regulatory element-binding protein in the regulation of adipogenesis. J Biol Chem 279: 23908–23915.

Katayama Y, Akatsu T, Yamamoto M, Kugai N, Nagata N. 1996. Role of nonenzymatic glycosylation of type I collagen in diabetic osteopenia. J Bone Miner Res 11: 931–937.

Kemink SA, Hermus AR, Swinkels LM, Lutterman JA, Smals AG. 2000. Osteopenia in insulin-dependent diabetes mellitus; prevalence and aspects of pathophysiology. J Endocrinol Invest 23:295–303.

Krakauer JC, McKenna MJ, Buderer NF, Rao DS, Whitehouse FW, Parfitt AM. 1995. Bone loss and bone turnover in diabetes. Diabetes 44:775–782.

Kreisberg JI, Radnik RA, Ayo SH, Garoni J, Saikumar P. 1994. High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cell cultures. Kidney Int 46: 105–112.

Kwon HM, Handler JS. 1995. Cell volume regulated transporters of compatible osmolytes. Curr Opin Cell Biol 7:465–471.

Kwon HM, Yamauchi A, Uchida S, Preston AS, Garcia-Perez A, Burg MB, Handler JS. 1992. Cloning of the cDNa for a Na^+/myo-inositol cotransporter, a hypertonicity stress protein. J Biol Chem 267:6297–6301.

Larkins RG, Dunlop ME. 1992. The link between hyperglycaemia and diabetic nephropathy [published erratum appears in Diabetologia 1992 Nov;35(11):1100]. Diabetologia 35:499–504.

Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV. 1994. Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: In situ localization of its receptor and c-fos messenger ribonucleic acids. Endocrinology 134:441–450.

Levin ME, Boisseau VC, Avioli LV. 1976. Effects of diabetes mellitus on bone mass in juvenile and adult-onset diabetes. N Engl J Med 294:241–245.

Lewis ML, Hughes-Fulford M. 2000. Regulation of heat shock protein message in Jurkat cells cultured under serum-starved and gravity-altered conditions. J Cell Biochem 77:127–134.

Li DQ, Chen Z, Song XJ, Luo L, Pflugfelder SC. 2004. Stimulation of matrix metalloproteinases by hyperosmolarity via a JNK pathway in human corneal epithelial cells. Invest Ophthalmol Vis Sci 45:4302–4311.

Locatto ME, Abranzon H, Caferra D, Fernandez MC, Alloatti R, Puche RC. 1993. Growth and development of bone mass in untreated alloxan diabetic rats. Effects of collagen glycosylation and parathyroid activity on bone turnover. Bone Miner 23:129–144.

Lytle C, Forbush B, 3rd. 1996. Regulatory phosphorylation of the secretory Na-K-Cl cotransporter: Modulation by cytoplasmic Cl. Am J Physiol 270:C437–C448.

McCabe LR, Kockx M, Lian J, Stein J, Stein G. 1995. Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. Exp Cell Res 218:255–262.

McCabe LR, Banerjee C, Kundu R, Harrison RJ, Dobner PR, Stein JL, Lian JB, Stein GS. 1996. Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: Role of Fra- 2 and Jun D during differentiation. Endocrinology 137:4398–4408.

McCarthy AD, Etcheverry SB, Bruzzone L, Cortizo AM. 1997. Effects of advanced glycation end-products on the proliferation and differentiation of osteoblast-like cells. Mol Cell Biochem 170:43–51.

McCarthy AD, Etcheverry SB, Cortizo AM. 2001. Effect of advanced glycation endproducts on the secretion of insulin-like growth factor-I and its binding proteins: Role in osteoblast development. Acta Diabetol 38:113–122.

McNair P, Christiansen C, Christensen MS, Madsbad S, Faber OK, Binder C, Transbol I. 1981. Development of bone mineral loss in insulin-treated diabetes: A 1 1/2 years follow-up study in sixty patients. Eur J Clin Invest 11:55–59.

Meyer HE, Tverdal A, Falch JA. 1993. Risk factors for hip fracture in middle-aged Norwegian women and men. Am J Epidemiol 137:1203–1211.

Munoz-Torres M, Jodar E, Escobar-Jimenez F, Lopez-Ibarra PJ, Luna JD. 1996. Bone mineral density measured by dual X-ray absorptiometry in Spanish patients with insulin-dependent diabetes mellitus. Calcif Tissue Int 58:316–319.

Nadkarni V, Gabbay KH, Bohren KM, Sheikh-Hamad D. 1999. Osmotic response element enhancer activity. Regulation through p38 kinase and mitogen-activated extracellular signal-regulated kinase kinase. J Biol Chem 274:20185–20190.

O'Donnell ME. 1993. Role of Na-K-Cl cotransport in vascular endothelial cell volume regulation. Am J Physiol 264:C1316–C1326.

O'Donnell ME, Brandt JD, Curry FR. 1995. Na-K-Cl cotransport regulates intracellular volume and monolayer permeability of trabecular meshwork cells. Am J Physiol 268:C1067–C1074.

Obata T, Brown GE, Yaffe MB. 2000. MAP kinase pathways activated by stress: The p38 MAPK pathway. Crit Care Med 28:N67–N77.

Ontiveros C, McCabe LR. 2003. Simulated microgravity suppresses osteoblast phenotype, Runx2 levels and AP-1 transactivation. J Cell Biochem 88:427–437.

Ontiveros C, Irwin R, Wiseman RW, McCabe LR. 2004. Hypoxia suppresses runx2 independent of modeled microgravity. J Cell Physiol 200:169–176.

Park CW, Kim JH, Lee JH, Kim YS, Ahn HJ, Shin YS, Kim SY, Choi EJ, Chang YS, Bang BK, Lee JW. 2000. High glucose-induced intercellular adhesion molecule-1 (ICAM-1) expression through an osmotic effect in rat mesangial cells is PKC-NF- kappa B-dependent. Diabetologia 43:1544–1553.

Parker JC. 1993. In defense of cell volume? Am J Physiol 265:C1191–C1200.

Pechhold K, Patterson NB, Blum C, Fleischacker CL, Boehm BO, Harlan DM. 2001. Low dose streptozotocin-induced diabetes in rat insulin promoter-mCD80-transgenic mice is T cell autoantigen-specific and CD28 dependent. J Immunol 166:2531–2539.

Pi M, Quarles LD. 2005. Osteoblast calcium-sensing receptor has characteristics of ANF/7TM receptors. J Cell Biochem 95:1081–1092.

Piepkorn B, Kann P, Forst T, Andreas J, Pfutzner A, Beyer J. 1997. Bone mineral density and bone metabolism in diabetes mellitus. Horm Metab Res 29:584–591.

Roger F, Martin PY, Rousselot M, Favre H, Feraille E. 1999. Cell shrinkage triggers the activation of mitogen-activated protein kinases by hypertonicity in the rat kidney medullary thick ascending limb of the Henle's loop. Requirement of p38 kinase for the regulatory volume increase response. J Biol Chem 274:34103–34110.

Sasaki T, Kaneko H, Ramamurthy NS, Golub LM. 1991. Tetracycline administration restores osteoblast structure and function during experimental diabetes. Anat Rec 231:25–34.

Schwartz AV, Sellmeyer DE, Ensrud KE, Cauley JA, Tabor HK, Schreiner PJ, Jamal SA, Black DM, Cummings SR. 2001. Older women with diabetes have an increased risk of fracture: A prospective study. J Clin Endocrinol Metab 86:32–38.

Selvamurugan N, Chou WY, Pearman AT, Pulumati MR, Partridge NC. 1998. Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence. J Biol Chem 273:10647–10657.

Semenza GL. 2000. HIF-1: Using two hands to flip the angiogenic switch. Cancer Metastasis Rev 19:59–65.

Sheikh-Hamad D, Di Mari J, Suki WN, Safirstein R, Watts BA, 3rd, Rouse D. 1998. p38 kinase activity is essential for osmotic induction of mRNAs for HSP70 and transporter for organic solute betaine in Madin-Darby canine kidney cells. J Biol Chem 273:1832–1837.

Shyng YC, Devlin H, Sloan P. 2001. The effect of streptozotocin-induced experimental diabetes mellitus on calvarial defect healing and bone turnover in the rat. Int J Oral Maxillofac Surg 30:70–74.

Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M. 1996. Transcriptional control of osteoblast growth and differentiation. Physiol Rev 76:593–629.

Steinbrech DS, Mehrara BJ, Saadeh PB, Chin G, Dudziak ME, Gerrets RP, Gittes GK, Longaker MT. 1999. Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro. Plast Reconstr Surg 104:738–747.

Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. 1983. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198.

Szkudelski T. 2001. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50:537–546.

Thomas DM, Hards DK, Rogers SD, Ng KW, Best JD. 1996a. Insulin receptor expression in bone. J Bone Miner Res 11:1312–1320.

Thomas DM, Maher F, Rogers SD, Best JD. 1996b. Expression and regulation by insulin of GLUT 3 in UMR 106-01, a clonal rat osteosarcoma cell line. Biochem Biophys Res Commun 218:789–793.

Trogan E, Choudhury RP, Dansky HM, Rong JX, Breslow JL, Fisher EA. 2002. Laser capture microdissection analysis of gene expression in macrophages from atherosclerotic lesions of apolipoprotein E-deficient mice. Proc Natl Acad Sci USA 99:2234–2239.

Tuominen JT, Impivaara O, Puukka P, Ronnemaa T. 1999. Bone mineral density in patients with type 1 and type 2 diabetes. Diabetes Care 22:1196–1200.

Uchida S, Kwon HM, Yamauchi A, Preston AS, Marumo F, Handler JS. 1992. Molecular cloning of the cDNA for an MDCK cell Na(+)- and Cl(−)-dependent taurine transporter that is regulated by hypertonicity. Proc Natl Acad Sci USA 89:8230–8234.

Verhaeghe J, van Herck E, Visser WJ, Suiker AM, Thomasset M, Einhorn TA, Faierman E, Bouillon R. 1990. Bone and mineral metabolism in BB rats with long-term diabetes. Decreased bone turnover and osteoporosis. Diabetes 39:477–482.

Verhaeghe J, Suiker AM, Visser WJ, Van Herck E, Van Bree R, Bouillon R. 1992. The effects of systemic insulin, insulin-like growth factor-I and growth hormone on bone

growth and turnover in spontaneously diabetic BB rats. J Endocrinol 134:485–492.

Verhaeghe J, Van Herck E, van Bree R, Moermans K, Bouillon R. 1997. Decreased osteoblast activity in spontaneously diabetic rats. In vivo studies on the pathogenesis. Endocrine 7:165–175.

Winchester SK, Selvamurugan N, D'Alonzo RC, Partridge NC. 2000. Developmental regulation of collagenase-3 mRNA in normal, differentiating osteoblasts through the activator protein-1 and the runt domain binding sites. J Biol Chem 275:23310–23318.

Wiske PS, Wentworth SM, Norton JA, Jr., Epstein S, Johnston CC, Jr. 1982. Evaluation of bone mass and growth in young diabetics. Metabolism 31:848–854.

Wolf BA, Williamson JR, Easom RA, Chang K, Sherman WR, Turk J. 1991. Diacylglycerol accumulation and microvascular abnormalities induced by elevated glucose levels. J Clin Invest 87:31–38.

Wolff SP, Dean RT. 1987. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. Biochem J 245:243–250.

Zayzafoon M, Stell C, Irwin R, McCabe LR. 2000. Extracellular glucose influences osteoblast differentiation and c-Jun expression. J Cell Biochem 79:301–310.

Zayzafoon M, Botolin S, McCabe LR. 2002. P38 and activating transcription factor-2 involvement in osteoblast osmotic response to elevated extracellular glucose. J Biol Chem 277:37212–37218.