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Glucose-Dependent Regulation of Osteoclast H^+-ATPase Expression: Potential Role of p38 MAP-Kinase

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Abstract Bone resorption is glucose concentration dependent. Mechanisms regulating glucose-dependent increases in bone resorption have not been identified. Glucose activates p38 MAP-kinase in other cells and since MAP kinases activate transcription factors, we hypothesized that glucose-stimulated bone resorption may be modulated by increased expression of the vacuolar H^+-ATPase. Glucose activates osteoclast p38 MAP-kinase in a time and concentration-dependent manner as determined by Western analysis with phospho-specific p38 antibody while total p38 levels are unchanged. The $K_{0.5}$ for glucose-dependent activation of p38 MAP-kinase is ~7 mM, activation is maximal at 30 min and is elevated but returning to basal levels by 60 min. The concentration-dependent increase in H^+-ATPase expression was confirmed by Northern analysis. The specific inhibitor of p38 MAP-kinase, SB203580, inhibited glucose transport in osteoclasts, as well as glucose concentration-dependent increases in bone resorption and expression of H^+-ATPase A and B subunits. Glucose had no effect on calmodulin expression levels that are regulated in response to other environmental changes. The glucose-stimulated increase in H^+-ATPase mRNA expression is a specific response to glucose since glucose has little effect on G3PDH mRNA levels. We conclude that glucose regulates osteoclast H^+-ATPase expression by a mechanism likely to involve p38 MAP-kinase. J. Cell. Biochem. 87: 75–84, 2002.
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Key words: bone; metabolism; diabetes; gene expression

Glucose homeostasis changes with age and glucose intolerance and insulin resistance are both correlated with increasing age [Preuss, 1997]. Bone loss in Type I diabetes results in diabetic osteopenia [Kayath et al., 1994], while Type II diabetics usually present clinically with increased bone mass. The latter is likely to occur due to the stimulatory effects of increased body mass on osteoblast activity [Krakauer et al., 1995; Haffner and Bauer, 1993].

Osteoclasts are multi-nucleated cells that form a sealed compartment on bone surfaces. HCl is secreted, via a vacuolar H^+-ATPase abundantly expressed in the ruffled membrane, into the resorption lacunae. Acid secretion degrades the mineral phase of bone [Blair et al., 1986, 1989; Teitelbaum, 2000] while the collagen matrix is degraded by acid proteases also secreted by osteoclasts [Teitelbaum, 2000]. Osteoclastic bone resorption is glucose concentration-dependent and activity is modulated within the physiologic range of glucose (3–8 mM) [Williams et al., 1997]. Glucose-dependent increases in osteoclastic bone resorption may play an important role in diabetic osteopenia and age-dependent bone loss. The aim of this study was to identify signaling pathways mediating glucose-dependent increases in bone resorption.

Glucose activates p38 MAP-kinase in many cell lines including glucose-responsive islet β-cells [MacFarlane et al., 1997; Igarashi et al., 1999; Natarajan et al., 1999]. p38 MAP-kinase is activated in response to osmotic stress by specific dual phosphorylation on threonine

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and tyrosine residues by upstream activators, MKK3 or MKK6 [Derijard et al., 1995]. p38 MAP-kinase mediates many diverse cellular processes including cytoskeletal rearrangement [Hedges et al., 1999], glucose transport [Goule et al., 1995; Sweeney et al., 1999], and alterations in gene expression [MacFarlane et al., 1997; Brinkman et al., 1999; Nadkarni et al., 1999]. Translocation of p38 MAP-kinase to the nucleus alters gene expression by activating specific transcription factors [MacFarlane et al., 1997; Bode et al., 1999] and specific promoter segments critical for glucose-dependent gene transcription, have been identified [Rufo et al., 2001]. Glucose mediates expression of multiple genes [Koo and Towle, 1999; Vaulont et al., 2000] and, in glucose-responsive cells like islet β-cells, stimulates expression of immediate early genes such as c-*fos* [Susini et al., 1998; Jonas et al., 2001].

Cytoskeletal rearrangement is critical for osteoclast motility and function [Teitelbaum, 2000]. Insertion of the vacuolar H^+-ATPase into the ruffled membrane of osteoclasts requires microtubule rearrangement [Lee et al., 1999a] and is likely to be energy-dependent. Interaction of the H^+-ATPase with the osteoclast actin cytoskeleton [Lee et al., 1999a] suggested that modulation of H^+-ATPase activity may be regulated, in part, by cytoskeletal rearrangement.

Glucose transport is essential for osteoclast activity due to the high-energy requirements of bone resorption and transport by osteoclasts cultured on bone is approximately twice that of bone [Williams et al., 1997]. Studies using the p38 inhibitor, SB203580, have implicated p38 MAP kinase in regulating glucose transport activity in other cell lines [Goule et al., 1995; Sweeney et al., 1999]. These studies demonstrate that p38 MAP-kinase is activated in response to increasing concentrations of glucose and that SB203580 inhibits osteoclast glucose transport, glucose-stimulated bone resorption, and glucose-dependent increases in H^+-ATPase expression. The glucose-stimulated increase in H^+-ATPase expression is specifically regulated by glucose since there is little effect on G3PDH mRNA levels. We conclude that elevated glucose levels regulate increased expression of the vacuolar H^+-ATPase thereby increasing bone resorption activity by osteoclasts by a mechanism that appears to involve p38 MAP-kinase.

MATERIALS AND METHODS

Osteoclast Purification and Culture

Osteoclast-rich cell fractions were obtained from medullary bone of laying hens on a Ca^{2+} restricted diet. Cell sedimentation through 70% serum results in 85–90% of cell nuclei in osteoclasts. Cells were cultured on 20–40 μm diameter bone fragments and incubated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% serum at 37°C in humidified air with 5% CO_2 as previously described [Blair et al., 1986; Williams et al., 1996a, 1997; Dong et al., 1999].

Cell Culture and Lysis

In short-term assays (glucose transport and p38 MAP-kinase activation), osteoclasts were incubated in glucose-free DMEM supplemented with serum (dialyzed to remove glucose) for 2 h prior to the experimental protocol. Cells cultured overnight were incubated in media with varying concentrations of D-glucose, washed with phosphate buffered saline and solubilized with lysis buffer as previously described [Dong et al., 1999]. Briefly, the buffer contains a cocktail of kinase, phosphatase, and protease inhibitors. The lysates were collected on ice and centrifuged at 14,000*g* for 10 min.

Bone Resorption Assay

Bone degradation by avian osteoclasts was quantified by direct radiometric assay using 100 μg of rat bone (20–40 μm fragments) labeled in vivo with L-[2,3,4,5-^{3}H]-proline as previously described [Williams et al., 1996a, 1997]. [^{3}H]-proline released into the medium was measured following 2 days in culture relative to no-cell controls eliminating the potential for artifacts due to macrophage contamination. Substrate specific activity (determined by substrate hydrolysis in 6 N HCl at 60°C, for 18 h) was used to determine micrograms of bone resorbed. Methods to eliminate artifacts have been described [Williams et al., 1997] and were utilized.

Western Analysis

Osteoclast lysates (25 μg protein) were resolved by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Non-specific binding was blocked with 2% bovine serum albumin, 0.1% gelatin in tris-buffered saline (TBS) for 1 h. Blots were incubated with primary antibody (anti-phospho-p38

antibody, New England BioLabs, Beverly, MA; or anti-H^+-ATPase A subunit, generous gift from Jan Mattson, AstraZeneca Pharmaceuticals, Molndal, Sweden) diluted 1:1,000 in TBS containing 0.05% Tween-20 (TBST20) overnight at 4°C. Blots were washed with TBST20 and incubated with secondary antibody for 1 h, washed again and immunoreactivity detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol.

Northern Analysis

Total RNA was isolated with Trizol reagent (Gibco/Life Technologies, Grand Island, NY) on ice. RNA, was precipitated with isopropyl alcohol and washed with 75% ethanol. Thirty micrograms of RNA from each treatment was resolved by electrophoresis on 1% agarose gels containing 2.2 M formaldehyde, 0.02 M MOPS pH 7.0, 8 mM sodium acetate, and 1 mM EDTA. RNA was transferred to Nytran SuperCharge membrane (Schleicher & Schuell, Keene, NH) by rapid downward capillary transfer. A 621 bp fragment of H^+-ATPase A subunit was amplified from the cDNA (generously provided by Dr. Roland Baron, Yale University, New Haven, CT) and used as a probe for Northern analysis. Primers (Integrated DNA Technologies, Coralville, IA) used for amplification of the probe were (forward) 5′-CCG TGA TGA GGA CAG AGA AG-3′ and (reverse) 5′-GGA CGG ACT TGA CGT ACA GG-3′. The probe was gel purified and labeled with Random Primers DNA Labeling System (Gibco/Life Technologies) incorporating [^{32}P]-dCTP. Hybridization was performed overnight at 42°C in buffer containing 50% formamide, 0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 2 × Denhardt's Reagent, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Blots were exposed to film overnight at −80°C.

Confocal Immunofluorescence Microscopy

Osteoclasts were cultured on glass cover slips and treated for varying lengths of time with 10 mM glucose. Cover slips were washed with PBS and fixed in 3% paraformaldehyde prior to being permeabilized with methanol and blocked. Cover slips were incubated with phospho-p38 antibody followed by incubation with FITC-conjugated secondary antibody in the dark. Cells were Hoescht stained for nuclear localization, mounted, and confocal fluorescence microscopy performed on a Leica Wetzler microscope.

Glucose Transport Assay

Cells were incubated in media containing 10 μM glucose and serum-starved for 1 h. Glucose transport was measured as accumulation of [^{3}H]-2-deoxyglucose (2-DOG) (American Radiolabeled Chemicals, Inc., St. Louis, MO), a non-metabolizable glucose analog, in 15 min assays, as previously described [Williams et al., 1997]. The effect of SB203580 on the concentration-dependence of glucose transport was tested by adding inhibitor 15 min prior to the initiation of the assay. Reactions were terminated by washing cells with ice cold PBS and lysed with 1 M NaOH. Lysates were neutralized with an equal volume of 1 M HCl. Transport was quantified by liquid scintillation spectrometry.

Metabolic Labeling and Immunoprecipitation

Osteoclasts were incubated in DMEM plus 10% serum containing the indicated concentrations of glucose ± 10 μM SB203580 for 24 h. Fresh media was added containing 0.25 mCi/ml [^{35}S]-methionine/cysteine and the indicated concentrations of glucose and the cells cultured ± SB203580 for an additional 24 h. Cells were lysed in buffer with 1% polyoxyethylene 9 lauryl ether in place of Triton X-100 [Dong et al., 1999]. H^+-ATPase subunits were immunoprecipitated from equal amounts of protein (100 μg) for 2 h with a monoclonal antibody to the H^+-ATPase (generous gift from Michael Forgac, Tufts University, Boston, MA). The immune complexes were precipitated with Protein A-Sepharose beads and washed four times with lysis buffer and resolved on 10% SDS–PAGE. Gels were stained, destained, and treated with EN^{3}HANCE autoradiography enhancer (NEN Life Science Products, Inc., Boston, MA), dried and exposed to X-ray film for 3 days at −80°C.

RESULTS

Bone resorption is glucose concentration-dependent with maximal resorption at ~7 mM glucose (Fig. 1). Osteoclasts were cultured with the indicated concentrations of D-glucose, and total hexose concentration was balanced with L-glucose, which is not transported by osteoclasts [Williams et al., 1997]. The data demonstrate that bone resorption, by isolated osteoclasts, is dynamically regulated in the physiological concentration range of glucose.

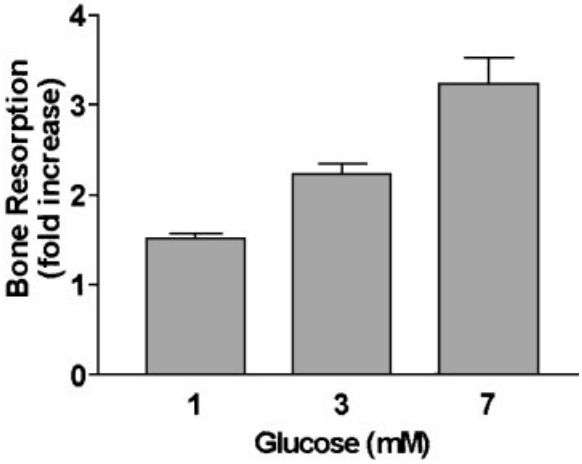

Fig. 1. Effect of glucose concentration on osteoclastic bone resorption. Bone degradation by avian osteoclasts was quantified by radiometric assay at the indicated concentrations of D-glucose as described in Materials and Methods. [^{3}H]-proline released into the medium by osteoclasts from labeled bone was measured following 2 days in culture relative to no-cell controls as described in Materials and Methods. The results are representative of six separate experiments each performed in quadruplicate, expressed as fold increase over control activity (0.3 mM glucose) ± SEM.

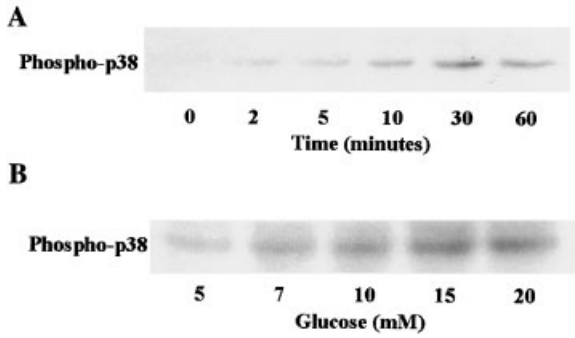

Fig. 2. Glucose-dependent activation of p38 MAP-kinase. (**A**) Cells were starved of glucose for 2 h followed by incubation with 10 mM D-glucose for the indicated times prior to lysis. Osteoclasts were washed and lysed as described in Materials and Methods. Solubilized lysates, normalized for protein, were resolved by SDS–PAGE, transferred to PVDF membrane, and probed with phospho-specific p38 antibody (New England Biolabs). Data are representative of three separate experiments. (**B**) Cells were incubated with 0.3 mM glucose for 2 h followed by incubation with the indicated concentrations of glucose for 30 min prior to lysis and Western analysis. Blots were probed with phospho-p38 antibody. Data are representative of two separate experiments.

Since p38 MAP-kinase mediates changes in gene expression in other cells [MacFarlane et al., 1997; Brinkman et al., 1999; Nadkarni et al., 1999] and is activated by osmotic stress [Kyriakis and Avruch, 1996; Bode et al., 1999], we analyzed the effects of glucose on the activation of p38 MAP-kinase in osteoclasts by Western analysis with the phospho-specific p38 MAP-kinase antibody (Fig. 2). Cells were treated for the indicated times with 10 mM D-glucose, the cells lysed, and 25 μg of total protein was resolved by 10% SDS–PAGE as described in Materials and Methods. Glucose activates p38 MAP-kinase in a time-dependent manner as determined by Western analysis with phospho-p38 antibody (Fig. 2A). No changes were observed in the amount of total p38 MAP-kinase (not illustrated). Maximal effects of glucose are observed at 30 min and are declining toward basal levels at 60 min. Parallel experiments were performed to determine whether other MAP kinase family members were activated in response to glucose and p38 MAP-kinase was the only MAP kinase activated (not illustrated). The concentration dependence for glucose-dependent activation of p38 MAP kinase was determined following 30 min treatment with the indicated concentrations of glucose (Fig. 2B). The $K_{0.5}$ for p38 MAP kinase activation was ~7 mM glucose with maximal effects at 15 mM glucose.

The effect of glucose on the apparent activation of p38 MAP-kinase was verified by confocal immunofluorescence microscopy on fixed and permeabilized osteoclasts (Fig. 3). Immunofluorescence microscopy indicates time-dependent changes in phospho-p38 MAP-kinase, similar to that observed in Western analysis on whole cell lysates (Fig. 2A). Minimal phospho-p38 MAP-kinase is evident in the absence of glucose stimulation. Diffuse cytoplasmic staining is evident at 5 min becoming increasingly perinuclear with maximal fluorescence at 30 min and declining at 60 min of treatment, (compare Figs. 2A and 3). The cells were Hoescht stained (blue) to show nuclei. The data support the hypothesis that glucose activates p38 MAP-kinase, stimulating translocation of p38 MAP-kinase to the nucleus.

Since glucose activates p38 MAP-kinase we examined the role of p38 MAP-kinase in glucose-dependent osteoclastic bone resorption by using SB203580, a selective inhibitor of p38 MAP-kinase (Fig. 4). Osteoclasts were incubated in the presence or absence of 10 μM SB203580 at the indicated concentrations of glucose and

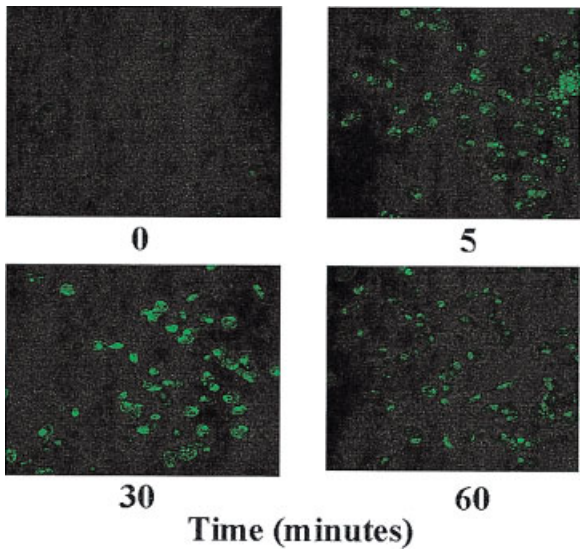

Fig. 3. Immunofluorescence localization of phospho-p38 MAP-kinase in osteoclasts. Cells attached to cover slips were starved of glucose for 2 h followed by incubation with 10 mM glucose for the indicated times. Cover slips were fixed, permeabilized, blocked, and incubated with phospho-p38 antibody and FITC-conjugated secondary antibody (green) as described in Materials and Methods. Cells were Hoescht stained for nuclei (blue). Data are representative of three separate experiments. [Color figure can be viewed in the online issue at www.interscience.wiley.com]

bone resorption was measured. SB203580 inhibited glucose-stimulated bone resorption 50% without altering basal bone resorption levels (0.3 mM glucose). Cells were examined daily to ensure cell viability and cell death was not observed.

We have previously shown that bone resorption is glucose concentration-dependent. Since glucose activates p38 MAP-kinase in osteoclasts and p38 MAP-kinase has been shown to mediate glucose transport in other cells, we hypothesized that p38 MAP-kinase may modulate glucose transport, and hence energy availability, in osteoclasts. We tested the effects of SB203580 on the concentration-dependence of glucose transport (Fig. 5). SB203580 inhibits the V_{max} of glucose transport by ~50% without significant effects on the $K_{0.5}$ (~2 mM) for transport. This data appear similar to the effect of SB203580 on glucose-stimulated bone resorption and suggest that glucose transport is essential for bone resorption by osteoclasts and is likely modulated by p38 MAP-kinase.

Since glucose alters the expression of multiple genes [Koo and Towle, 1999; Vaulont et al., 2000], we hypothesized that one potential mechanism of glucose-dependent increases in bone resorption was glucose-dependent modulation of H^+-ATPase expression. We tested the effects of increasing concentrations of glucose on expression of the osteoclastic H^+-ATPase (Fig. 6). Osteoclasts were cultured with the indicated concentrations of glucose for 18 h, and the cells washed and lysed. Western analysis of

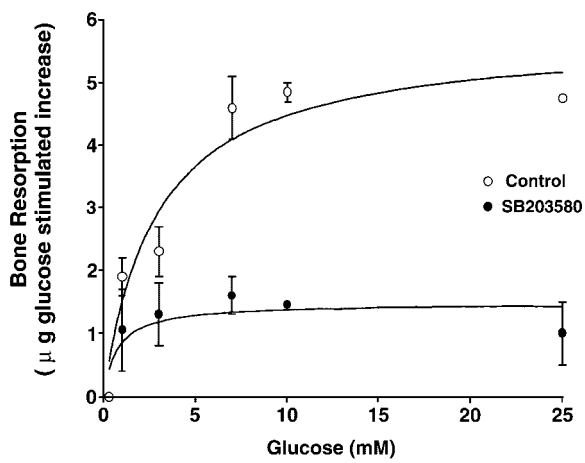

Fig. 4. Effect of SB203580 on glucose-stimulated bone resorption. Bone resorption was measured at the indicated concentrations of D-glucose in the absence or presence of the p38 MAP-kinase inhibitor, SB203580 (10 μM). Label released into the media was quantified by scintillation spectrometry following 2 days in culture. Data are the mean ± SEM of three separate experiments each performed in quadruplicate.

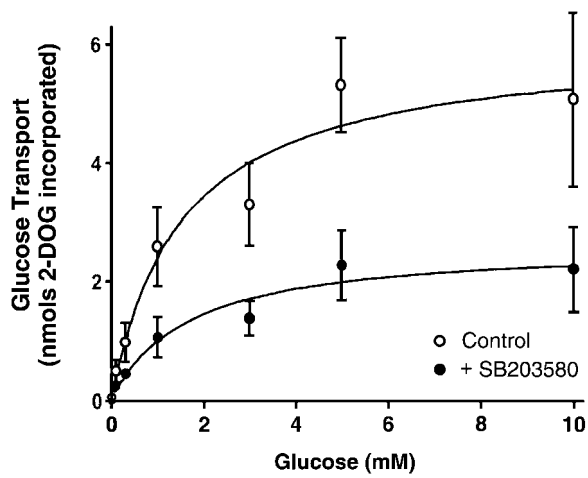

Fig. 5. Effect of SB203580 on glucose transport. The glucose concentration dependence of glucose transport was measured by [^{3}H]-2-deoxyglucose accumulation in osteoclasts at the indicated glucose concentrations ± SB203580. Transport was measured for 15 min and terminated following washing by lysis and neutralization. Label uptake was quantified by scintillation spectrometry. Data are the mean ± SEM of three separate experiments each performed in triplicate.

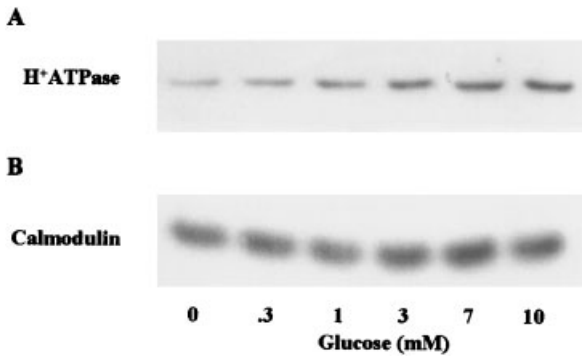

Fig. 6. Glucose concentration-dependent changes in the A subunit of the H^+-ATPase. (**A**) Osteoclasts were incubated with the indicated concentrations of D-glucose for 18 h prior to lysis. Solubilized lysates (25 μg protein) were resolved on 10% SDS–PAGE, transferred to PVDF membrane and probed with a polyclonal antibody to the 70 kDa subunit of the H^+-ATPase. (**B**) Western analysis performed on cell lysates from osteoclasts treated with the indicated concentrations of glucose and blots probed with monoclonal antibody to calmodulin. Data are representative of three separate experiments.

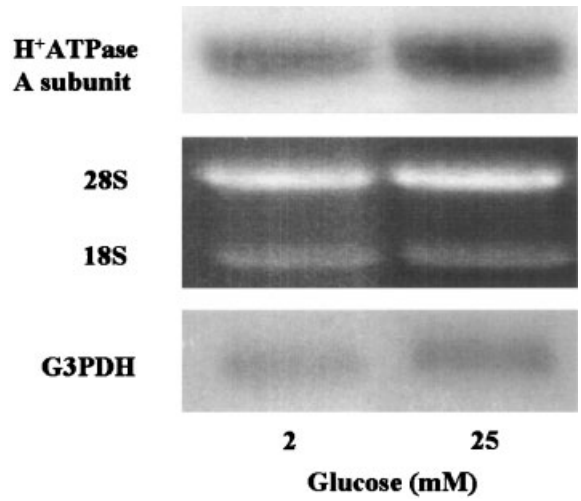

Fig. 7. Glucose-stimulated up-regulation of H^+ATPase mRNA expression. Osteoclasts were incubated for 24 h in 2 or 25 mM glucose. Total RNA was isolated with Trizol Reagent. 30 μg of RNA was separated by formaldehyde denaturing gel electrophoresis, transferred to nylon membrane and probed for the A subunit of the H^+ATPase (**top panel**). Ethidium bromide stained RNA before transfer is shown in **middle panel**. Membrane was stripped and probed for G3PDH as shown in the **bottom panel** (n=3).

lysates using a polyclonal antibody to the 70 kDa A subunit of the H^+-ATPase (generous gift from Jan Mattson, AstraZenaca Pharmaceuticals, Sweden) demonstrates that glucose up-regulates the A subunit of the H^+-ATPase in a concentration-dependent manner with maximal effects at 10 mM glucose (Fig. 6A), while the expression of calmodulin does not change (Fig. 6B). The fact that calmodulin levels are unchanged in response to glucose suggests that the increase in H^+-ATPase is a selective glucose-stimulated effect since calmodulin levels are modulated in osteoclasts in response to other stimuli [Williams et al., 1996b, 2000].

To determine the mechanism by which glucose regulates H^+-ATPase expression, Northern analysis was performed with a probe generated from the A subunit cDNA of the avian osteoclastic H^+-ATPase on total RNA isolated from osteoclasts cultured in low (2 mM) or high (25 mM) glucose for 24 h (Fig. 7, top panel) as described in Materials and Methods. High glucose results in a greater than twofold increase in A subunit mRNA. The ethidium bromide-stained gel is shown to demonstrate equal loading and quality of the RNA (Fig. 7, middle panel). This observation suggests that glucose tightly regulates H^+-ATPase mRNA expression. Glucose had little effect on G3PDH gene expression (Fig. 7, bottom panel), suggesting that the observed changes in H^+-ATPase expression are specifically regulated in response to glucose.

The role of p38 MAP-kinase in H^+-ATPase expression was further examined by testing the effect of SB203580 on the glucose concentration-dependent increases in H^+-ATPase expression in metabolic labeling experiments. Osteoclasts were cultured as described in Materials and Methods and the effects of SB203580 on the expression of the osteoclastic H^+-ATPase determined by [^{35}S]-methionine labeling and immunoprecipitation. H^+-ATPase expression was visualized by fluorographic enhancement and autoradiography (Fig. 8). H^+-ATPase expression, in the absence of SB203580, is stimulated in a glucose concentration-dependent manner with maximal levels at 10 mM glucose, which is consistent with data in Figure 6. Under the conditions utilized, multiple subunits of the H^+-ATPase are immunoprecipitated [Myers and Forgac, 1993]. SB203580 blocked the glucose concentration-dependent up-regulation in both A and B subunits of the H^+-ATPase. A parallel non-immune immunoprecipitation (NI) from 25 mM glucose minus SB203580 is shown as control. This data suggests that p38 MAP-kinase is a key regulator of glucose-stimulated increases in H^+-ATPase expression.

DISCUSSION

Previous work has shown that SB203580, the inhibitor of p38 MAP-kinase, preserved bone mineral density and decreased bone resorption in mice [Badger et al., 1996]. SB242235, a newer p38 MAP kinase inhibitor, has also been shown to protect bone tissue and preserve bone mineral density in a rat model of adjuvant-induced arthritis [Badger et al., 2000]. While the mechanisms involved in these studies were not conclusive, since cells other than osteoclasts may have been affected, the data were consistent with effects on p38 MAP-kinase. SB203580 inhibited IL-1-stimulated bone resorption by isolated osteoclasts in neonatal rabbit bone pit assays [Ranges et al., 1999] and both compounds dose dependently inhibit p38 MAP-kinase activity in fetal bone rudiments and IL-1 stimulated bone resorption [Kumar et al., 2001]. These data support a role for p38 MAP-kinase in modulating bone resorption and are consistent with our findings that SB203580 inhibits glucose-stimulated bone resorption in isolated osteoclasts.

Mechanisms regulating glucose concentration-dependent increases in bone resorption were investigated. Glucose initiates a series of signaling events [Larsen et al., 1999] and stimulates osteoclastic bone resorption [Williams et al., 1997]. In the earlier studies measuring the glucose concentration dependence of bone resorption, it was necessary to balance hexose concentrations with a non-transportable hexose (L-glucose). Failure to balance the hexose concentration results in a concentration dependence curve that does not plateau (not illustrated). While it is necessary to balance the hexose concentration in the experimental setting, in order to eliminate osmotic gradient differences as a variable, this suggests that bone resorption would continue to increase in a case of worsening hyperglycemia. We hypothesized that since glucose activates a cascade of short-term signaling events, this would result in changes in gene expression thereby altering osteoclastic activity. The role of MAP-kinase activation in glucose-dependent changes in osteoclast activity was tested. Isolated osteoclasts were challenged with glucose and p38 MAP-kinase was the only MAP kinase family member activated in response to glucose as determined by Western analysis and immunofluorescence microscopy (Figs. 2 and 3). Activation of p38 MAP-kinase was maximal at 30 min and declines at 60 min but remained above basal levels. The time course of changes in phospho-p38 MAP-kinase levels was similar in both sets of experiments. The $K_{0.5}$ for activation of p38 MAP-kinase, determined from 30 min dose experiments, is $\sim$7 mM which is well within the physiologic concentration range of glucose. Immunofluorescent localization data suggest that activated p38 MAP-kinase localizes to the nucleus as expected. The fact that changes in glucose concentration modulate p38 phosphorylation in the physiologic range of glucose suggests a role for p38 MAP-kinase in glucose-dependent signaling in osteoclasts.

p38 MAP-kinase is an important signaling molecule in osteoclastogenesis [Matsumoto et al., 2000a,b]. However, much less is known about the role of p38 MAP-kinase in modulating the activity of mature osteoclasts. SB203580, the specific inhibitor of p38 MAP-kinase, is widely used to evaluate the involvement of p38 MAP-kinase in cellular physiology [Wilson et al., 1997; Kumar et al., 1999]. The reported IC_{50} of SB203580 for p38 MAP-kinase is 0.6 μM [Cuenda et al., 1995]. SB203580 competitively inhibits p38 MAP-kinase by binding the enzyme's ATP-binding site [Young et al., 1997]. Our data indicate that p38 MAP-kinase is involved in modulating glucose transport (Fig. 5), although the precise role in regulating glucose transport is not known. SB203580 (10 μM) inhibits glucose transport in osteoclasts in 15 min assays suggesting that p38 MAP-kinase likely plays a role in the regulation of osteoclast activity by altering early glucose-dependent signaling events. In view of the glucose concentration dependence of bone resorption, inhibition of glucose transport with SB203580 likely inhibits glucose-dependent bone resorption by decreasing the energy resources available for bone resorption [Williams et al., 1997] (Fig. 4).

Recently, SB203580 was shown to inhibit JNK2β1 and c-Raf [de Laszlo et al., 1998; Lee et al., 1999b]. SB242235, another selective inhibitor of p38 MAP-kinase has been employed to verify the results obtained with SB203580 and had nearly identical effects on inhibition of p38 MAP-kinase and bone resorption [Kumar et al., 2001]. ERK, in a c-Raf-dependent manner, and JNK are known to be activated in response to glucose in other cell types [Bandyopadhyay et al., 2000; Purves et al., 2001], but we were unable to detect phospho-ERK or phospho-JNK

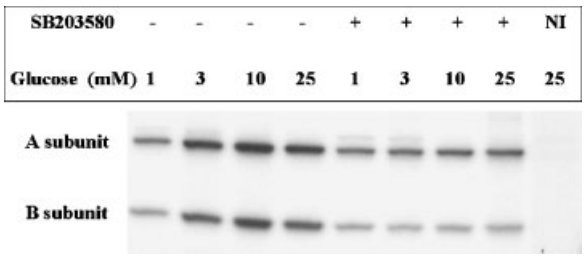

Fig. 8. Effect of SB203580 on glucose-stimulated up-regulation of H^+-ATPase subunits. Osteoclasts were [^{35}S]-methionine labeled in culture at the indicated glucose concentrations for 24 h followed by lysis and immunoprecipitation of H^+-ATPase subunits and autoradiography as described in Materials and Methods. [^{35}S]-methionine-labeled proton pump was visualized by autoradiography. Data are representative of two separate experiments.

levels in osteoclasts challenged with glucose. Since p38 MAP-kinase was the only MAP kinase family member activated in response to glucose and SB203580 only inhibited glucose-stimulated changes in activity, it is unlikely that the effects of SB203580 are due to effects on a kinase other than p38. This data support a role for p38 MAP-kinase in mediating metabolic changes in osteoclast activity.

We hypothesized that glucose stimulates changes in osteoclast H^+-ATPase gene expression. The data demonstrate that glucose up-regulates H^+-ATPase mRNA and protein levels in osteoclasts in a dose-dependent manner as determined by Western and Northern analysis (Figs. 6 and 7). Furthermore, the glucose-stimulated increase in H^+-ATPase mRNA expression is regulated specifically by glucose since glucose does not significantly alter G3PDH mRNA levels. Glucose-stimulated increases in H^+-ATPase mRNA and protein levels suggest that H^+-ATPase expression is dynamically regulated by glucose. Inhibition of glucose-stimulated H^+-ATPase protein expression by SB203580 (Fig. 8) suggests a role for p38 MAP-kinase in mediating glucose-stimulated bone resorption.

Previous work has demonstrated that bone resorption is glucose concentration-dependent [Williams et al., 1997]. We have extended the earlier observations in this study demonstrating that glucose modulates signaling in osteoclasts leading to increased H^+-ATPase mRNA and protein levels. Taken together, these data suggest that osteoclast activity and vacuolar H^+-ATPase expression levels are coupled to fuel availability. Others have reported a direct interaction between the glycolytic enzyme, aldolase, and the H^+-ATPase [Lu et al., 2001] and hypothesized that the interaction could provide a mechanistic link between glucose metabolism and proton pumping. The apparent glucose responsiveness of osteoclasts, demonstrated here, supports this hypothesis.

p38 MAP-kinase mediates changes in gene expression by translocating to the nucleus upon activation (Fig. 3) and activating specific transcription factors. This study demonstrates that glucose activates p38 MAP-kinase signaling in the short-term and prolonged exposure to elevated glucose concentrations increases H^+-ATPase expression. The p38 inhibitor, SB203580, inhibits glucose-stimulated bone resorption, glucose transport, and up-regulation of H^+-ATPase expression. We conclude that glucose concentration-dependent changes in osteoclastic bone resorption are mediated by regulation of H^+-ATPase expression, and that these changes are modulated, at least in part, by p38 MAP-kinase.

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