Insulin-like growth factor I rapidly enhances acid efflux from osteoblastic cells

ANU SANTHANAGOPAL AND S. JEFFREY DIXON
Department of Physiology and Division of Oral Biology, School of Dentistry, Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1

Santhanagopal, Anu, and S. Jeffrey Dixon. Insulin-like growth factor I rapidly enhances acid efflux from osteoblastic cells. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E423–E432, 1999.—Insulin-like growth factor I (IGF-I) is thought to stimulate bone resorption indirectly through a primary effect on osteoblasts, which in turn activate osteoclasts by as-yet-unidentified mechanisms. Small decreases in extracellular pH (pH\textsubscript{o}) dramatically increase the resorptive activity of osteoclasts. Our purpose was to characterize the effect of IGF-I on acid production by osteoblastic cells. When confluent, UMR-106 osteoblast-like cells and rat calvarial cells acidified the compartment beneath them. Supplementation with IGF-I caused a further decrease in pH\textsubscript{o}. To investigate the mechanism, we monitored acid efflux from subconfluent cultures. IGF-I rapidly increased net efflux of H\textsuperscript{+} equivalents in a concentration-dependent manner. IGF-II (10 nM) evoked a smaller response than IGF-I (10 nM). The response to IGF-I was partially dependent on extracellular Na\textsuperscript{+}, but not glucose, and exhibited little if any desensitization. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase abolished the response to IGF-I but not to parathyroid hormone. Thus IGF-I enhances acid efflux from osteoblastic cells, via a signaling pathway dependent on activation of phosphatidylinositol 3-kinase. In vivo, acidification of the compartment between the osteogenic cell layer and the bone matrix may affect diverse processes, including mineralization and osteoclastic bone resorption.

extracellular pH; osteoblasts; microphysiometry; phosphatidylinositol 3-kinase; wortmannin

REMOLDING OF THE SKELETON is a complex process regulated by interactions among bone cells, extracellular matrix, and soluble factors. Insulin-like growth factors (IGFs) IGF-I and IGF-II are among the most abundant growth factors present in the bone matrix and play important roles in skeletal growth and development. IGF-I stimulates the proliferation of preosteoblastic cells, the synthesis of collagen by mature osteoblasts, and the formation of bone in vivo (8, 9).

In contrast to the well-characterized anabolic actions of IGF-I, its effects on bone resorption are less well understood. Parenteral administration of IGF-I to human subjects rapidly increases serum and urinary markers of bone resorption and formation (12, 16). In addition, IGF-I stimulates osteoclast formation in organ cultures of mouse metacarpals/metatarsals (27) and in cultures of mouse hematopoietic blast cells (18). Furthermore, IGF-I stimulates resorption by mixed populations of mouse or rabbit bone cells cultured on dentin slices (14, 18). Similarly, when rodent bone cells were cultured on ivory slices for 24 h, IGFs increased both the number and volume of osteoclastic resorption lacunae (15). Interestingly, IGF-I has no effect on the resorptive activity of isolated rodent osteoclasts, but responsiveness to IGFs is restored when osteoclasts are cocultured with osteoblastic cells. Thus IGFs appear to stimulate the resorptive activity of osteoclasts directly via a primary effect on other cell types such as osteoblasts (15).

Resorptive agents (such as parathyroid hormone) act on osteoblasts, which in turn stimulate osteoclast formation and activity. This process may involve enhanced osteoblastic expression of cell-surface signaling molecules or release of soluble mediators. In this regard, decreasing extracellular pH (pH\textsubscript{o}) markedly enhances osteoclastic resorption (1, 7, 26). In vitro, even slight acidification of the culture medium can dramatically increase the number of resorption pits formed by osteoclasts (2). Thus osteoblast-mediated acidification of their environment may rapidly increase the resorptive activity of mature osteoclasts (1).

The purpose of our study was to determine the effects of IGF-I on acid efflux from osteoblastic cells by use of a Cytosensor microphysiometer to monitor real time changes in pH\textsubscript{o} beneath osteoblastic cells. We examined the ability of osteoblastic cells to acidify an extracellular compartment, characterized the effects of IGF-I on this process, and investigated the underlying signaling pathways.

METHODS

Materials and solutions. α-Minimum essential medium (α-MEM, cat. no. 12571) buffered with HCO\textsubscript{3}{−} (26 mM), phosphate-buffered saline (cat. no. 14040), heat-inactivated fetal bovine serum (FBS, cat. no. 26140), antibiotic solution (penicillin 10,000 units/ml; streptomycin 10,000 μg/ml; and amphotericin B 25 μg/ml, cat. no. 15240), trypsin solution (nominally Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free, 0.05% trypsin, and 0.53 mM EDTA (cat. no. 25300)), Dulbecco’s modified Eagle’s medium (DMEM, cat. no. 23800), and HCO\textsubscript{3}{−}-free MEM (used in standard superfusion medium, cat. no. 41500) were obtained from Gibco Laboratories (Burlington, ON, Canada). Bovine albumin (fraction V, fatty acid free) was from Boehringer Mannheim (Laval, QC, Canada; cat. no. 775835). Collagen type II (cat. no. C-6885), 2-deoxyglucose, N-methyl-D-glucamine (NMG), and cytochalasin B were obtained from Sigma (St. Louis, MO). IGF-I and -II (recombinant human, cat. nos. H-3102 and H-7020, respectively) and parathyroid hormone (PTH) fragment [human, PTH-(1–34), cat. no. H-4835] were obtained from Bachem (Torrance, CA). Stock solutions of IGF-I and -II were prepared in standard superfusion medium (see next paragraph) and stored in aliquots in −20°C. For experiments investigating the dependence of
proton efflux on extracellular glucose or Na⁺, a stock solution of IGF-I was prepared in Na⁺- and glucose-free buffer and stored in aliquots at −80°C. A stock solution of PTH-(1—34) was prepared in 0.005 N acetic acid with 1 mg/ml bovine albumin and stored in aliquots at −80°C. Phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors, wortmannin, and LY-294002 (2-(4-morpholino)-8-phenyl-4H-1-benzo pyran-4-one) were obtained from Calbiochem (La Jolla, CA). Stock solutions of wortmannin and LY-294002 were prepared in dimethyl sulfoxide and stored in aliquots at −80°C. 2-Deoxy-o-[1,2-N-H]glucose (26 Ci/mmol) was purchased from Du Pont Canada (Lachine, Q.C.).

Standard superfusion medium was HCO₃⁻-free MEM supplemented with HEPES (1 mM) and bovine albumin (1 mg/ml) adjusted to pH 7.30 ± 0.02 and osmolarity of 290 ± 5 mosM/l. Glucose dependence was investigated using DMEM supplemented with HEPES (1 mM), L-glutamine (2 mM), bovine albumin (1 mg/ml) with or without glucose (5.5 mM, glucose-containing and glucose-free medium, respectively), adjusted to pH 7.30 ± 0.02 and 290 ± 5 mosM/l. Na⁺-dependence experiments were performed using Na⁺-containing and Na⁺-free buffers containing (in mM): 4.4 KCl, 1 KH₂PO₄, 0.8 MgSO₄, 1 HEPES, 1.8 CaCl₂, 5.5 glucose, and 150 either NaCl or NMG chloride supplemented with bovine albumin (1 mg/ml), and adjusted to pH 7.30 ± 0.02 and 290 ± 5 mosM/l. The buffering power (b) of each of these solutions was determined using a Mettler DL21 titrator (Hightstown, N.J.) over a pH range of 7.0–7.3 at 37°C. The values of b in mM/pH unit were: standard superfusion medium, 1.4 ± 0.3; Na⁺-containing medium, 1.2 ± 0.1; glucose-containing medium, 1.3 ± 0.1; Na⁺-containing buffer, 1.1 ± 0.2; and Na⁺-free buffer, 3.0 ± 0.3 (n = 3).

Cells and culture. The clonal osteoblast-like cell line UMR-106, originally isolated from a rat osteosarcoma (20), was obtained from the American Type Culture Collection (Rockville, MD). These cells exhibit properties of osteoblasts, including type I collagen production, high alkaline phosphatase activity, responsiveness to PTH and IGF-I, and formation of mineralized tumors in rats (20, 22). UMR-106 cells were subcultured twice weekly in α-MEM supplemented with FBS (10% vol/vol) and antibiotics (1% vol/vol).

An osteoblast-like rat bone cell population was obtained from neonatal rat calvaria with a modification of the technique of Pockwinse et al. (21). Wistar rats (1–7 days of age) were killed by decapitation. The frontal and parietal bones were dissected free of periosteum and other soft tissues. Calvarial fragments from 3–5 animals were digested in 5 ml trypsin solution for 10 min at 37°C. The supernatant was discarded, and the semidigested fragments were transferred to 0.2% collagenase in HCO₃⁻-free MEM for 30 min at 37°C. The supernatant was discarded, and calvarial fragments were digested again with 0.2% collagenase. After 60 min, supernatant containing released cells was removed and mixed with an equal volume of FBS to halt further enzyme activity. The resultant cell suspension was sedimented, and the pellet was resuspended in α-MEM supplemented with FBS (10% vol/vol) and antibiotics (1% vol/vol). Cells were seeded on 60-mm culture dishes (Becton-Dickinson, Rutherford, N.J.) at a density of 2 × 10⁴ cells/cm². Medium was replaced after 24 h. Primary cultures were grown for ~1 wk until confluent and then were used for experiments.

UMR-106 or primary rat calvarial cells were harvested by exposure to trypsin solution and then seeded on porous polycarbonate membranes (Transwell, 12 mm diameter, 3 μm pore size, Costar, Corning, NY) in α-MEM containing HCO₃⁻ (26 mM) supplemented with FBS (10% vol/vol) and antibiotics (1% vol/vol). Cultures were grown in humidified 5% CO₂-95% air at 37°C for 48 h. Supplemented medium was replaced with serum-free α-MEM for the final 24 h before experiments. Cells were seeded at low (7.5–15 × 10⁴ cells/cm²) or high (30 × 10⁴ cells/cm²) density for experiments monitoring the changes in pH₀ beneath the cell layers. For experiments monitoring rates of acidification, cells were seeded at low density only.

For glucose uptake studies, UMR-106 cells were seeded (5 × 10⁴ cells/cm²) on 60-mm culture dishes in α-MEM supplemented with FBS (10% vol/vol) and antibiotics (1% vol/vol) and grown for 48 h. Medium was replaced with serum-free α-MEM supplemented with bovine albumin (1 mg/ml) for the final 24 h before experiments. Measurement of pH₀ and acidification rate. Cells adhering to the polycarbonate membranes were placed in microflow chambers and positioned above silicon-based potentiometric sensors, which can detect changes in pH₀ of as little as 10⁻⁷ units (Cytosensor microphysiometer, Molecular Devices, Sunnyvale, CA) (17). Cells were continuously superfused at a rate of 100 µl/min with the indicated solution at 37°C. Superfusion solutions with low buffering power were used to enhance the changes in pH₀ resulting from small alterations in efflux of H⁺ from cells. Each of the four microflow chambers was supplied with superfusion solution from one of two reservoirs. Flow was regulated by a computer-controlled valve, and the lag between a valve switch and the arrival of solution at the microflow chamber was 4–5 s. The surface potential of each silicon sensor, corresponding to the pH₀, was plotted initially as a voltage-time trace. At 37°C, a change of 61 mV corresponds to a change in pH₀ of 1 unit. During experiments in which the effects of IGF-I on pH₀ were monitored, cells were superfused continuously with standard medium or IGF-I (in standard medium).

To measure the rate of acidification, fluid flow to cells was stopped periodically for 22 or 30 s. During this time, acids accumulating in the microflow chamber (volume 2.8 µl) caused pH₀ to decrease. Measurement of acidification rate was performed by linear least square fit to the slope of the voltage-time trace during the time when fluid flow to the cells was stopped. Net cellular efflux of H⁺ equivalents (proton efflux) was calculated from the acidification rate based on the buffering power of the superfusion solution and volume of the microflow chamber.

At the end of certain experiments, cells were superfused with Triton X-100 (0.1% in standard medium) to permeabilize cells and allow determination of the absolute pH₀ beneath high-density cell layers. To test for possible nonspecific interactions of test substances with the silicon sensor, cultures were rendered nonviable by superfusion with detergent solution and then superfused with test substances and solutions.

Scanning electron and light microscopy. The preparation of specimens for scanning electron microscopy was based on techniques of Brunk et al. (6). Following selected experiments, membranes with adherent cells were removed from the microflow chambers and placed in fixative (3% glutaraldehyde in 0.1 M cacodylate buffer) at room temperature for 1 h. The membranes were then rinsed in cacodylate buffer and postfixed (1% OsO₄ in cacodylate buffer) for 1 h. Membranes were subsequently rinsed in water and dehydrated through a graded series of ethanol. Specimens were then critical point-dried and coated with gold-palladium (8 nm) in a vacuum. Membranes were examined using a Hitachi 35 scanning electron microscope operated at 20 kV. In some other experi-

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¹ These procedures were approved by the Council on Animal Care of The University of Western Ontario.
ments, cultures were fixed in ethanol, embedded in paraffin, sectioned perpendicular to the membrane, stained with hematoxylin and eosin, and examined by light microscopy.

Measurement of glucose uptake. Initial rates of glucose uptake by UMR-106 cells were measured using radiolabeled 2-deoxyglucose, as described previously (22). Cultures were treated with IGF-I (10 nM) or vehicle in conditioned serum-free α-MEM for 15 min at 37°C in 5% CO2-95% air. Cultures were then washed and incubated for 1 min at 23°C with 2-deoxy-o-[3H]glucose (60 µM, specific activity adjusted with unlabeled 2-deoxyglucose to 3.3 mCi/mmol) in transport buffer. Transport buffer consisted of (in mM) 134 NaCl, 5.4 KCl, 1.8 CaCl2, 0.8 MgSO4, and 20 HEPES, adjusted to pH 7.30 ± 0.02 and 290 ± 5 mosM/l. Where indicated, cytochalasin B (10 µM) was included in the transport buffer to inhibit facilitated hexose transport. An aliquot of incubation buffer was collected at the end of each uptake incubation. Incubations were terminated by washing cultures with ice-cold isosmotic Tris-sucrose solution. Cells were harvested by osmotic lysis (1 ml water/dish) and mechanical scraping. An aliquot (100 µl) of the cell harvest was used for protein measurement, and the remainder was combined with scintillation cocktail. The radioactive contents of the buffer and cells were measured using liquid scintillation counting.

Statistics. Acidification rates were normalized as a percentage of basal rates in standard superfusion medium before addition of test substance or change of superfusion solution. This normalization compensated for differences in cell numbers among the four chambers. Results are presented as representative traces or as means ± SE. Comparisons between two means were performed using the Student's t-test. Comparisons among three or more means were performed by ANOVA followed by a Tukey-Kramer test for multiple comparisons. Differences were accepted as statistically significant at P < 0.05.

RESULTS

Effects of IGF-I on pHo beneath UMR-106 osteoblast-like cells. UMR-106 cells were cultured at high or low density on porous polycarbonate membranes. Cultures were placed in microflow chambers, and pHo was monitored using sensors located beneath the membranes. In this set of experiments, cultures were continuously superfused with standard medium. IGF-I (10 nM in standard medium) induced a rapid decrease in pHo beneath high-density cultures (the maximum decrease was 0.07 ± 0.01 pH units below basal, n = 19) (Fig. 1A). This drop in pHo was sustained for the remainder of the 21-min exposure to IGF-I. Upon washout of IGF-I, pHo slowly recovered, returning to basal levels within ~1 h. In contrast, superfusion of low-density cultures with IGF-I, or high-density cultures with vehicle, did not cause any significant change in pHo (as summarized in Fig. 1B).

In high-density cultures, there was a sustained decrease in pHo in the continued presence of IGF-I (Fig. 2, A and B). Prolonged exposure to IGF-I (10 nM for 1–2 h) resulted in a prolonged decrease in pHo (0.07 ± 0.01 pH units, n = 8). Responses to multiple exposures to IGF-I were also examined (Fig. 2C). An initial exposure of high-density cells to IGF-I (10 nM, 21 min) decreased pHo by 0.06 ± 0.02 units (n = 4) and then, following a 1-h wash, a second exposure decreased pHo by 0.06 ± 0.02 units (n = 4). These data indicate that, under the conditions used, there is little if any desensitization of the acidification response.

The basis for the different responses of low- and high-density cultures to IGF-I was investigated using scanning electron microscopy. Following selected experiments, in which IGF-I-induced pHo changes were monitored, membranes were carefully removed from the microflow chambers, fixed, and prepared for scanning electron microscopy. As expected, low-density cultures were subconfluent, and numerous open pores were apparent (Fig. 3A). In contrast, high-density cultures were confluent, and open pores were observed rarely (Fig. 3B). Histological sections revealed that pores were indeed patent in low-density, but not in high-density, cultures (not shown). It is likely that, in low- and high-density cultures, H+ efflux is directed both downward across the membrane and upward into the superfusion solution. However, in low-density cultures, the H+ equivalents directed downward can diffuse rapidly through the patent pores into the superfusion solution. In contrast, in high-density cultures, H+ equivalents directed downward likely accumulate beneath the cell layer. It is conceivable that acid accumulates beneath high-density cultures, even under basal conditions. To examine this possibility and to allow
determination of the absolute pHo beneath high-density cultures, selected cultures were superfused with Triton X-100 (0.1%). As expected, this caused a shift in pHo to that of the standard superfusion medium (pH 7.3). From the magnitude of this shift, the absolute value of basal pHo beneath high-density cultures was calculated to be 7.02 ± 0.05 (n = 4).

Effect of IGF-I on proton efflux from UMR-106 osteoblast-like cells. Next, we considered the possibility that the effects of IGF-I on pHo were due to increased efflux of H+ equivalents after stimulation of cells by growth factor. For these and all subsequent studies, acidification rate was determined from the rate of decrease in pHo when superfusion of the cultures was periodically interrupted, and this value was used to calculate proton efflux. Superfusion of cells with IGF-I (10 nM) caused a rapid increase in proton efflux from both high- and low-density cultures. However, changes in proton efflux caused by the associated drop in pHo made data recorded from high-density cultures difficult to interpret. Therefore, low-density cultures were used for the detailed studies of proton efflux presented below. The increase in proton efflux from low-density cultures was sustained in the presence of IGF-I (Fig. 4A). IGF-I (10 nM) induced a maximum increase in proton efflux of 16 ± 1% above basal levels (n = 27). In contrast, superfusion of cells with vehicle did not change proton efflux (Fig. 4A). In standard superfusion medium, basal acidification rates were 0.076 ± 0.004 pH units/min (n = 111), which correspond to a proton efflux per cell sample of 0.30 ± 0.02 nmol/min.

Effect of multiple IGF-I treatments on proton efflux was also assessed (Fig. 4B). An initial application of IGF-I (10 nM) caused a sustained increase in proton efflux (10 nM IGF-I was sufficient to induce a maximum response; Fig. 5). Upon washout of IGF-I, proton efflux

Furthermore, the rapid initial drop in pHo introduced an artifact, which caused an apparent enhancement of the initial increase in proton efflux induced by IGF-I.
proton efflux from nonvital cells was zero. Further-

superfusion with detergent. As expected, the basal solution with the silicon sensor, cells were killed by were artifacts arising from direct interaction of the test B

tion. Low-density cultures of UMR-106 cells were superfused with standard medium, and acidification rates were monitored. Cell samples were superfused with the indicated concentrations of IGF-I in standard medium for 20–21 min (as shown in Fig. 4A for 10 nM IGF-I). Data are maximum increase in proton efflux above basal levels, expressed as a percentage of basal. Values are means ± SE of 3–27 samples for each point from ≥3 separate cell preparations. Sigmoid curve was fit by nonlinear regression using Prism (Graph-Pad Software, San Diego, CA). Mean value of basal proton efflux per sample was 0.35 ± 0.04 nmol/min (n = 53).

slowly returned toward basal levels (within ~1 h). Subsequent application of IGF-I (10 nM) induced an increase in proton efflux comparable to that of the initial response (the maximum magnitude of the second response was 86 ± 16% of the initial response, n = 4), indicating little desensitization. Thus the pattern of changes in proton efflux induced by IGF-I mirrors the changes in pHo induced by IGF-I (e.g., compare Figs. 2C and 4B).

To rule out the possibility that responses to IGF-I were artifacts arising from direct interaction of the test solution with the silicon sensor, cells were killed by superfusion with detergent. As expected, the basal proton efflux from nonvital cells was zero. Furthermore, no changes were observed upon superfusion with IGF-I, indicating that neither the change in pHo nor the increase in proton efflux was an artifact.

Evidence for involvement of the IGF-I receptor. To investigate the pathway underlying the effect of IGF-I on proton efflux from UMR-106 cells, we first examined its dependence on IGF-I concentration. An increase in proton efflux was observed at IGF-I concentrations ≥10 pM. The concentration of IGF-I required to elicit a half-maximal response (EC50) was ~300 pM, with maximum increase in proton efflux occurring at 10–30 nM IGF-I (Fig. 5). The concentration dependence of this response is in keeping with the values of EC50 reported for responses mediated by the IGF-I receptor in other systems (29).

Second, we compared the acidification responses to IGF-I (10 nM) and IGF-II (10 nM), both of which activate the IGF-I receptor. IGF-II caused a sustained increase in proton efflux from UMR-106 cells, with a time course similar to that induced by IGF-I (Fig. 6). However, the magnitude of the response to IGF-II was significantly less than the response to IGF-I (9 ± 1% and 16 ± 1%, respectively). These findings are consistent with the greater effect of IGF-I compared with IGF-II in other bone systems (8, 15, 18) and with the involvement of the IGF-I receptor in mediating changes in proton efflux.

Dependence of IGF-I-induced acidification response on extracellular Na+. We next investigated the mechanisms underlying the IGF-I-induced increase in proton efflux from UMR-106 cells. Na+/H+ exchange, mediated by NHE-1, is an important pathway for proton efflux in osteoblasts (13). To investigate the involvement of Na+-dependent processes such as NHE-1, we compared the response of cells to IGF-I (10 nM) in the presence and absence of extracellular Na+. Cells were initially superfused with standard medium. When cells were then superfused with control (Na+-containing) buffer, IGF-I induced a maximum increase in proton efflux of 27 ± 4% above basal (n = 5; Table 1). When parallel cell samples were superfused with nominally Na+-free buffer, the basal proton efflux dropped to 91 ± 8% of

![Fig. 5. Dependence of increase in proton efflux on IGF-I concentration. Low-density cultures of UMR-106 cells were superfused with standard medium, and acidification rates were monitored. Cell samples were superfused with the indicated concentrations of IGF-I in standard medium for 20–21 min (as shown in Fig. 4A for 10 nM IGF-I). Data are maximum increase in proton efflux above basal levels, expressed as a percentage of basal. Values are means ± SE of 3–27 samples for each point from ≥3 separate cell preparations. Sigmoid curve was fit by nonlinear regression using Prism (Graph-Pad Software, San Diego, CA). Mean value of basal proton efflux per sample was 0.35 ± 0.04 nmol/min (n = 53).](http://ajpendo.physiology.org/)

![Fig. 6. Comparison of effects of IGF-I and IGF-II on proton efflux. A: low-density cultures of UMR-106 cells were superfused with standard medium, and acidification rates were monitored. Proton efflux was calculated from acidification rate and expressed as a percentage of basal proton efflux. Where indicated by shaded area, cells were superfused with IGF-I (10 nM), IGF-II (10 nM), or vehicle (Veh) in standard medium. Responses are from parallel samples and are representative of responses from ≥3 separate cell preparations. Values of basal proton efflux per sample were (in nmol/min): Veh, 0.05; IGF-I, 0.14; IGF-II, 0.09. B: maximum increases in proton efflux (above basal levels) in response to IGF-I, IGF-II, or vehicle. Values are means ± SE of 11–27 samples for each bar, from ≥7 separate cell preparations. Mean values of basal proton efflux per sample were (in nmol/min): Veh, 0.19 ± 0.04, n = 11; IGF-I, 0.31 ± 0.04, n = 22; IGF-II, 0.27 ± 0.04, n = 12. *Response to IGF-I significantly different from response to IGF-II or vehicle (P < 0.05). #Response to IGF-II significantly different from response to IGF-I or vehicle (P < 0.05).](http://ajpendo.physiology.org/)
Table 1. Effects of extracellular Na\(^+\) and glucose on basal and IGF-I-stimulated proton efflux

<table>
<thead>
<tr>
<th>Superfusion Solution</th>
<th>Proton Efflux</th>
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<tr>
<td></td>
<td>Basal</td>
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<tr>
<td>Control (Na(^+)-containing)</td>
<td>100</td>
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<tr>
<td>Na(^+) free</td>
<td>91 ± 8</td>
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<tr>
<td>Control (Glucose containing)</td>
<td>100</td>
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<tr>
<td>Glucose free</td>
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Values are expressed as percentages of basal proton efflux in the appropriate control solution and are means ± SE of 3–5 samples from 3 separate cell preparations. Aciddification rates of low-density UMR-106 cell cultures were monitored using a microphysiometer. Cells were initially superfused with standard medium. For Na\(^+\)-dependence studies, cells were then superfused with control (Na\(^+\)-containing) or Na\(^+\)-free buffer (time 0–13.5 min). Basal protein efflux was calculated from unstimulated acidification rate at 10 min. Cells were then superfused with IGF-I (10 nM) in the appropriate buffer from 13.5 to 24 min. Maximum acidification rate during this period was used to calculate IGF-I-stimulated proton efflux. From 24 to 37.5 min, cells were superfused with appropriate buffer without IGF-I. At 37.5 min, cells were again superfused with standard medium. A similar protocol was used for glucose dependence studies except that, in some experiments, cells were superfused with control (glucose-containing) or glucose-free medium for longer periods of time. Values of basal proton efflux per sample were 0.11 ± 0.03 nmol/min (for control Na\(^+\)-containing buffer) and 0.26 ± 0.10 nmol/min (for control glucose-containing medium). *P < 0.05 for effect of removal of extracellular Na\(^+\) or glucose.

control levels, and IGF-I induced an increase in proton efflux of 15 ± 4% above basal (significantly less than the response to IGF-I in Na\(^+\)-containing buffer). When these cells were once again superfused with standard medium (which contains physiological [Na\(^+\)]\(_{i}\)), there was a transient overshoot in proton efflux before it returned to the level seen in control cells (i.e., cells exposed to IGF-I in Na\(^+\)-containing buffer). In summary, removal of Na\(^+\) had little effect on basal proton efflux but significantly suppressed the increase in acid efflux induced by IGF-I.

Role of glucose in IGF-I-induced acidification response. We considered the possibility that the IGF-I-induced increase in proton efflux was due to enhanced metabolism of glucose, perhaps secondary to an increase in glucose uptake. Thus we compared the acidification response of cells to IGF-I (10 nM) in the presence and absence of extracellular glucose. UMR-106 cells were initially superfused with standard medium. When cells were then superfused with control (glucose-containing) medium, IGF-I induced a maximum increase in proton efflux of 13 ± 2% above basal (n = 3; Table 1). When parallel cell samples were superfused with glucose-free medium, there was a large and rapid decrease in basal proton efflux to 23 ± 6% of control levels. This finding indicates that basal metabolic acid production by these cells is strongly dependent on glucose. However, in the absence of glucose, IGF-I still induced a marked increase in proton efflux of 12 ± 1% above basal (not significantly different from the response to IGF-I in control glucose-containing medium). When the cells were once again superfused with glucose-containing standard medium, proton efflux returned slowly to basal levels. In summary, although removal of extracellular glucose caused a large, reversible decrease in basal proton efflux, IGF-I still induced a substantial increase in proton efflux.

IGF-I, like insulin, increases facilitated hexose transport activity in a number of cell types, including osteoblasts (22, 25). Therefore, we considered the possibility that the IGF-I-induced increase in proton efflux was due to an increase in the rate of glucose uptake. To investigate the effect of IGF-I on glucose uptake, we measured the initial rate of uptake of 2-deoxyglucose. UMR-106 cells were pretreated with IGF-I (10 nM) or vehicle for 15 min. Uptake was then assessed using 1-min incubations with 2-deoxy-[\(^3\)H]glucose in the presence or absence of the facilitated hexose transport inhibitor cytochalasin B (10 µM). As expected, cytochalasin B blocked glucose uptake. In contrast, IGF-I had no significant effect on the initial rate of 2-deoxyglucose uptake (Fig. 7). Taken together, these findings indicate that the IGF-I-induced increase in proton efflux does not arise simply from an increase in the rate of glucose uptake.

Role of PI 3-kinase in the IGF-I-induced acidification response. In other systems, PI 3-kinase mediates many of the metabolic responses to IGF-I (25). To assess the involvement of PI 3-kinase in the IGF-I-induced acidification response, we used wortmannin, a potent inhibitor of PI 3-kinase (10). UMR-106 cells were initially treated with wortmannin (100 nM) or vehicle in standard superfusion medium for 10 min. Wortmannin by itself did not change basal proton efflux. Cells were then treated with IGF-I (10 nM) for 20–21 min in the presence of wortmannin or vehicle. Under these conditions, wortmannin virtually abolished the IGF-I-induced increase in proton efflux from a maximum of 15 ± 3% in vehicle-treated control cells to 0.9 ± 0.6% in wortmannin-treated cells (n = 4, Fig. 8). No change in

![Fig. 7. Effect of IGF-I on glucose uptake. Low-density cultures of UMR-106 cells were pretreated with IGF-I (10 nM) or vehicle (Veh) for 15 min at 37°C. Initial rate of glucose uptake was determined by incubation with 2-deoxy-[\(^3\)H]glucose (60 µM) for 1 min at 23°C. Assays were performed in the absence (Control) or presence of cytochalasin B (10 µM, to inhibit facilitated hexose transport). Uptake rates are expressed as nmol 2-deoxyglucose · g cell protein\(^-1\) · min\(^-1\). Values are means ± SE from 4 separate experiments. Bars labeled with different lowercase letters are significantly different (P < 0.05).](http://ajpendo.physiology.org/doi/10.220.33.5.on June 25, 2017)
proton efflux was observed upon washout of wortmannin.

To investigate the possibility that wortmannin nonspecifically inhibited acid production or transport, we tested the effect of wortmannin on the response to PTH. PTH acts through a cAMP-dependent pathway to elevate proton efflux in UMR-106 cells (S. J. Harvey and S. J. Dixon, unpublished observations). In contrast to its effect on the IGF-I response, wortmannin (100 nM) had no significant effect on the response to PTH ([hPTH- (1—34)], 100 nM) (Fig. 8, B and C). Thus wortmannin does not inhibit the ability of these cells to produce or extrude acid after activation of PI 3-kinase-independent pathways.

We next investigated the effects of LY-294002, a specific PI 3-kinase inhibitor structurally unrelated to wortmannin (10). LY-294002 (50 µM) itself caused a reversible decrease in proton efflux to 39 ± 8% of basal efflux (n = 3), possibly due to nonspecific inhibition of metabolic acid production. Like wortmannin, LY-294002 virtually abolished the IGF-I-induced acidification response from a maximum of 25 ± 4% in vehicle-treated control cells to 0.6 ± 0.6% in cells treated with LY-294002 (n = 3). Taken together, these findings indicate a role for PI 3-kinase in mediating the effects of IGF-I on proton efflux from osteoblast-like cells.

Effect of IGF-I on pHo and proton efflux from osteoblasts isolated from rat calvaria. Responses to IGF-I were also examined using first-passage osteoblast-enriched cultures obtained from rat calvaria. When high-density calvarial cell cultures were continuously superfused with standard superfusion medium, IGF-I (10 nM) rapidly induced a sustained decrease in pHo beneath the cell layer (the maximum decrease was 0.025 ± 0.003 pH units below basal, n = 7) (Fig. 9A). Superfusion of high-density cultures with vehicle did not cause any significant change in pHo. Thus, as in
UMR-106 cells, IGF-I induced a sustained decrease in pHo beneath high-density cultures of calvarial cells.

We next investigated the effects of IGF-I on proton efflux using low-density calvarial cell cultures. Superfusion of cells with IGF-I caused a rapid increase in proton efflux, which was sustained in the presence of IGF-I and reached maximum levels of 13 ± 2% above basal (n = 10) (Fig. 9B). In contrast, superfusion of cells with vehicle did not change proton efflux. Thus the responses of UMR-106 cells to IGF-I correspond closely to those seen in first-passage calvarial cell cultures.

**DISCUSSION**

Confluent osteoblastic cells acidify the compartment beneath the cell layer. In the present study, we used a Cytosensor microphysiometer to monitor changes in pHo beneath cultures of UMR-106 osteoblast-like and rat calvarial cells. The compartment between confluent osteoblastic cell layers and the silicon sensor was acidified under basal conditions, and the pHo of this compartment was further decreased in response to IGF-I. Decreases in pHo were observed beneath confluent high-density cell layers but not beneath subconfluent low-density cell layers. This finding suggests that confluent layers act as barriers to diffusion, resulting in accumulation of protons beneath the cell layer. This model is in keeping with early suggestions that the "internal" pH of bone must be considerably lower than 7.4 (19).

The decrease in pHo induced by IGF-I may be due to an increase in net efflux of H+ equivalents. Alternatively, IGF-I may decrease the paracellular passage of H+ equivalents across the cell layer. However, IGF-I caused increases in proton efflux, which exhibited several similarities to the effect of IGF-I on pHo. These include a similar pattern, time course, and lack of desensitization, suggesting that the effect of IGF-I on pHo is due to an increase in proton efflux from the cells.

During exposure to IGF-I, a new steady-state pHo is attained when the increase in cellular H+ efflux becomes balanced by an increase in paracellular H+ diffusion. Alternatively, the new steady-state pHo may arise when the decrease in pHo inhibits further proton efflux. In this regard, it has been shown that lowering pHo reduces net H+ efflux via Na+/H+ exchange and inhibits production of lactic acid by cultured calvaria (13, 19).

The IGF-I receptor mediates IGF-I-induced increase in proton efflux. Our observations indicate involvement of the IGF-I receptor in mediating the rapid increase in proton efflux from osteoblast-like cells. The magnitude of this increase was dependent on the concentration of IGF-I. The EC50 for this response of 300 pM agrees well with the EC50 for responses mediated by the IGF-I receptor in other systems but is considerably lower than the EC50 for responses to IGF-I mediated through the insulin receptor (29). Furthermore, IGF-II (which activates the IGF-I receptor) caused an increase in proton efflux qualitatively similar to that induced by IGF-I. The smaller magnitude of the response to IGF-II is in keeping with the relative effects of IGF-I and IGF-II in other bone systems (8, 15, 18). Although both IGF-I and IGF II receptors are present on osteoblasts, the IGF-II receptor (the mannose 6-phosphate receptor) is not thought to mediate transmembrane signaling (8, 9). Thus the IGF-I receptor appears to mediate the effects of IGFs on proton efflux.

The increase in proton efflux and the associated decrease in pHo were sustained in the continued presence of IGF-I. Interestingly, washout of IGF-I was followed by recovery of both proton efflux and pHo to basal levels over a period of ~1 h. This time course is similar to that reported in rat fibroblasts for recycling of IGF-I receptors after their activation and internalization (31). In our system, a second comparable response to IGF-I could be elicited after a 1-h recovery. Taken together, these findings indicate that the acidification responses are sustained in the continued presence of IGF-I, are reversible upon washout, and do not exhibit short-term desensitization. Monitoring proton efflux may offer a convenient approach for studying the kinetics and regulation of desensitization to IGF-I and other ligands in osteoblasts and other cell types.

Signaling pathways and metabolic mechanisms underlying the effect of IGF-I on proton efflux. The IGF-I receptor is an αβ2 heterotetrameric receptor tyrosine kinase. Upon activation, the receptor autophosphorylates and also phosphorylates intracellular substrates such as the insulin receptor substrates and Shc. Several signaling pathways are then stimulated, including those activated by phospholipase C-γ, Ras/MAP kinase, and PI 3-kinase, a regulator of cellular metabolism. We investigated the role of PI 3-kinase in mediating the effects of IGF-I on proton efflux by use of pharmacological probes. Wortmannin is a potent inhibitor of PI 3-kinase, with little or no effect on PI 4-kinase, Src protein tyrosine kinase, protein kinase C, cAMP-dependent protein kinase, cGMP-dependent protein kinase, or calmodulin-dependent protein kinase II. However, wortmannin does inhibit phospholipase A2, mitogen-activated protein kinase, and myosin light-chain kinase, although markedly higher concentrations of wortmannin are required to inhibit the latter two enzymes than are required to inhibit PI 3-kinase (10, 25). In our study, we found that the effects of IGF-I on proton efflux were virtually abolished by wortmannin (100 nM), a concentration that selectively inhibits PI 3-kinase in other intact cell systems. The specificity of wortmannin was investigated using PTH, which signals through both adenylyl cyclase and phospholipase C-β. In UMR-106 cells, PTH enhances proton efflux through elevation of cAMP (S. J. Harvey and S. J. Dixon, unpublished data). The lack of effect of wortmannin on the increase in proton efflux induced by PTH argues against a nonspecific effect of this inhibitor on metabolic acid production.

To further assess the involvement of PI 3-kinase, we examined the effect of LY-294002, a less potent but highly specific inhibitor of PI 3-kinase, which is structurally unrelated to wortmannin. At a concentration that blocks PI 3-kinase in other systems, LY-294002 abolished the IGF-I-induced increase in proton efflux.
These findings indicate that the IGF-I acidification response is dependent on activation of PI 3-kinase.

In other systems, PI 3-kinase mediates acute metabolic responses to IGF-I and insulin (25). In skeletal muscle and adipocytes, PI 3-kinase stimulates glycogen synthesis by activation of glycogen synthase and increases glucose uptake by translocation of facilitated hexose transporters to the cell surface. In cardiac muscle, PI 3-kinase is thought to enhance glycolysis through activation of phosphofructokinase-2. Studies of energy metabolism in bone have shown high rates of anaerobic glycolysis, utilizing extracellular glucose and yielding lactic acid (19, 24). These findings are in keeping with our observation of a rapid drop of ~75% in basal proton efflux on removal of glucose from the extracellular medium. However, the IGF-I-induced increase in proton efflux was not dependent on extracellular glucose, implying the involvement of other pathways for metabolic acid production. This observation, together with the lack of an acute effect of IGF-I on glucose uptake, argue against the possibility that IGF-I acts by translocating facilitated hexose transporters to the plasma membrane.

The sustained nature of the IGF-I-induced increase in proton efflux is consistent with a net increase in metabolic acid production, rather than merely activation of proton efflux pathways. Had only acid extrusion mechanisms been activated, the response would have been transient, lasting only until a more alkaline steady-state intracellular pH was established. The metabolic mechanisms underlying the response to IGF-I remain unclear but may involve increased ATP hydrolysis, lipolysis, or glycogenolysis followed by glycolysis (producing lactic acid) or oxidative phosphorylation (producing carbonic acid).

There are a number of possible mechanisms for the efflux of H⁺ equivalents produced by IGF-I-induced metabolic changes. In several tissues, lactic acid efflux is mediated by a family of H⁺/monocarboxylate cotransporters (MCT1–3) (5), which may be present in bone. In addition, Na⁺/H⁺ exchange mediated by NHE-1 is a key pathway for H⁺ efflux from osteoblasts (3, 13). NHE-1 is known to be activated by a number of growth factors, and IGF-I has been reported to stimulate Na⁺/H⁺ exchange in rat vascular smooth muscle cells (28). Although removal of extracellular Na⁺ had little effect on basal proton efflux, it reduced the magnitude of the IGF-I-induced increase in proton efflux by ~45%. This finding is consistent with NHE mediating efflux of a portion of the acid produced in response to IGF-I. The overshoot observed upon reintroduction of extracellular Na⁺ suggests that IGF-I still increased the production of acid, despite acid efflux being temporarily suppressed by inactivation of Na⁺/H⁺ exchange.

Under the conditions used in our studies, there would be little if any HCO₃⁻ influx, because all superfusion solutions used in the microphysiometer must be nominally HCO₃⁻ free, to avoid the production of gas bubbles. However, it is possible that in vivo HCO₃⁻ influx contributes to the efflux of H⁺ equivalents under basal conditions, or during stimulation by IGF-I or other growth factors. In summary, IGF-I acts through the IGF-I receptor to activate PI 3-kinase and enhance metabolic acid production. This acid is then extruded by both Na⁺-dependent and Na⁺-independent mechanisms.

Possible physiological significance of proton efflux from osteoblastic cells. Acid production by cells of the osteoblast lineage may serve several functions. Small changes in pH₀ modify gap junctional communication (30) and expression of egr-1 and type 1 collagen (11) in cultured osteoblasts. When osteoblasts are actively synthesizing and secreting osteoid in vivo, it is possible that the pH₀ of the microenvironment between the osteoblast layer and the mineralizing front is regulated, affecting both the rate of mineral formation and phase transformation. An acidic zone beneath the active osteoblast layer may prevent mineralization of the osteoid seam during bone formation.

It is also possible that acid production by bone-lining cells and adjacent osteogenic cells activates osteoclastic bone resorption (1, 2, 7, 26). In vitro studies of pit formation by rat osteoclasts have shown that there is little, if any, resorptive activity at values of pH₀ > 7.3. Slight decreases in pH₀ markedly stimulate osteoclastic resorption, which is maximally active at pH₀ values < 7.0 (2). In our system, the compartment beneath confluent osteoblasts was acidified to a steady-state value 0.2–0.3 pH units below the pH of the superfusion medium. Furthermore, in response to IGF-I, osteoblasts acidified this compartment by an additional 0.05–0.1 pH units. Thus it is possible that localized acidification contributes to the stimulatory effects of IGF-I on resorption in vitro and in vivo. However, it is difficult to estimate the actual value of the local pH₀ changes in vivo, which will depend on proton efflux, buffering power, volume of the compartment, and permeability of the cell layer. The buffering power of extracellular fluid in vivo is greater than that of the superfusion media used in vitro. This would tend to make the changes in pH₀ in vivo smaller than those seen in vitro. On the other hand, the volume of the fluid compartment between the osteogenic cell layer and the bone matrix in vivo is likely very small, tending to enhance pH₀ changes in vivo. Although little is known about the permeability characteristics of the osteoblast layer in vivo, the presence of a “functional membrane or cellular envelope, which separates the extracellular fluids of bone from those of the animal as a whole” has been suggested by several authors (19).

Like IGF-I, PTH is thought to stimulate osteoclastic resorption indirectly through a primary effect on osteoblastic cells. It is noteworthy that PTH enhances proton efflux from UMR-106 cells (Fig. 8) and SaOS-2 human osteoblast-like cells (4). Thus local acidification may contribute to the resorptive effects of both IGF-I and PTH. It will be of interest to determine whether other resorptive agents also stimulate acid efflux from osteoblasts and to assess the contribution of local changes in pH₀ to the regulation of osteoclastic resorption in vivo.
In conclusion, IGF-I enhances acid production by osteoblastic cells via a signaling pathway dependent on activation of PI 3-kinase. The ensuing drop in pHo may then modulate important processes such as mineralization and osteoblastic resorption. In this regard, wortmannin has been shown to inhibit bone resorption in vitro and in vivo (23). It is possible that, in addition to wortmannin also inhibits resorption indirectly through suppression of acid production by osteoblasts.

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Address for reprint requests and other correspondence: S. J. Dixon, Dept. of Physiology, Faculty of Medicine and Dentistry, The Univ. of Western Ontario, London, Ontario, Canada N6A 5C1 (E-mail: jdxon@physiology.uwo.ca).

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