Endocrine, *vol. 13, no. 3, 345–352, December 2000* 0969–711X/00/13:345–352/$12.00

Real-Time Detection of Insulin-Like Growth Factor-1 Stimulation of the MAC-T Bovine Mammary Epithelial Cell Line

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Binding of growth factors by cell-surface receptors is an essential means by which cells regulate normal tissue growth and differentiation. Exposure to growth factors is often transient, and our goal was to determine whether short-term exposure to insulin-like growth factor-1 (IGF-1) would lead to activation, assayed as cell proliferation, of mammary epithelial cells. The MAC-T cell line is an immortalized bovine mammary epithelial cell line, chosen as our model mammary cell line because of its known sensitivity to IGF-1. Using the Cytosensor® Microphysiometer System, a biosensor capable of measuring extracellular acidification, we were able to measure activation of the cells owing to IGF-1 addition in real time and found that peak acidification occurred in only 14 min. We show that this rapid response to IGF-1 is dose dependent and specific for IGF-1. A significant increase in [^{3}H]thymidine incorporation by cells after a similar short-term exposure to IGF-1 suggests that the measured increase in extracellular acidification following IGF-1 addition is physiologically relevant. This technology offers a new, novel, and rapid means for the study of IGF-1 activity, as well as the screening of IGF-1 inhibitors, in mammary epithelial cells.

Key Words: Insulin-like growth factor-1; microphysiometer; mammary epithelial cells; MAC-T.

Introduction

Insulin-like growth factor-1 (IGF-1) plays a role in the normal function of the mammary gland, including the stimulation of mammary growth and the secretion of milk *(1)*. Recent reviews *(2–4)* provide evidence that IGF-1, supplied by the circulation or by local tissue synthesis, promotes mammogenesis. Forsyth et al. *(5)* and Hovey et al. *(2)* have provided direct evidence for synthesis of IGF-1 in the developing ovine mammary gland, and we *(6)* have reported that IGF-1 contributes to much of the mitogenic activity of bovine mammary tissue extracts. In addition, we have previously found that the synthesis of IGF-1 and IGF binding proteins (IGFBPs) in the prepubertal bovine mammary gland is affected by treatment with bovine somatotropin and by feeding *(7)*. Regardless of the IGF-1 source, the effects of IGF-1 on mammogenesis depend on the stimulation of cell proliferation and on the interaction of IGF-1 with receptors in the epithelium. Current cell culture methods of analyzing these interactions focus primarily on static culture assays that allow time for binding, downstream processing, and long-term activation. In vivo, fluid flow can alter cell exposure time to IGF-1. The development of new tools to analyze IGF-1 stimulation and screen potential IGF-1 inhibitors in real time that can be correlated to long-term effects such as cell proliferation are potentially valuable.

Cell activation by an extracellular growth factor such as IGF-1 is initiated by binding of the growth factor to a transmembrane receptor molecule *(8)*. The signal generated is specific to the activated receptor although there can be cross-reactivity between signaling ligands, as evidenced by IGF-1 and insulin, both of which can signal via the type I IGF-1 receptor (IGF-1R) and via the insulin receptor *(9)*. Both are heterotetrameric receptors composed of two extracellular α-subunits and two transmembrane β-subunits *(10)*. Despite structural similarities, there appear to be unique intracellular substrates for both IGF-1R and insulin receptor that likely impact the differences in activity *(9)*. However, both ligands can induce cell proliferation through the IGF-1R, although a higher concentration of insulin is required *(11)*. This difference in concentration is related to the different affinities of the two molecules for IGF-1R *(12)*. For IGF-1 signaling, ligand-receptor binding is further complicated by the availability of extracellular IGFBPs, which can alter binding dynamics *(13–15)*.

In the present study, we focused on the interaction of IGF-1 with the MAC-T cell line because of the importance of the IGF-1 axis on bovine mammary development. The MAC-T cell line was developed by transfecting primary bovine mammary epithelial cells with the SV-40 large T-antigen *(16)*. This cell line retains the hormonal responsiveness of the primary mammary cells and is responsive to IGF-1, but is not responsive to physiologic concentrations

Received May 4, 2000; Revised July 3, 2000; Accepted July 27, 2000.

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of insulin *(17,18)*. To investigate the real-time response of MAC-T cells to IGF-1, we chose to use the Cytosensor® Microphysiometer System (Molecular Devices, Sunnyvale, CA).

The microphysiometer is capable of detecting rapid binding of ligand to cell-surface receptors by detecting the extracellular acidification induced on receptor activation *(19)*. Living cells acidify the extracellular environment as a result of metabolic activity within the cells *(20)*. The microphysiometer is unique in that it can measure the extracellular pH change on-line and allows one to report measured response with little delay time. It is configured with four separate cell chambers and flow paths to allow the simultaneous measurement of four separate cell samples. Typically, a low-buffer media is pumped across a monolayer of living cells in 2-min cycles, and the pH is recorded each second throughout the cycle. Toward the end of each cycle, the pump is stopped, allowing the metabolic by-products from the cells to accumulate in the cell chamber. Accumulation of by-products resulting from to cell stimulation leads to a decrease in the pH of the cell chamber, which, owing to the small fluid volume (1.4 μL) in the cell chamber, can be quantified. This pH change is referred to as the extracellular acidification rate (ECAR). ECAR is essentially constant for equilibrated cells but is altered in the presence of stimulant.

Numerous studies have coupled changes in ECAR with the introduction of specific ligand *(21–23)*. Chan et al. *(21)* examined the stimulation of heregulin receptors by heregulin-α in the presence of HER2 and HER3 in human mammary and ovarian carcinoma cells. In a study by Pitchford et al. *(22)*, the stimulation of tyrosine kinase receptors for nerve growth factor by nerve growth factor resulted in a detectable response that was dose dependent. Similarly, autocrine stimulation by transforming growth factor-α in B82 mouse fibroblast cells was recently examined and a reduction in ECAR owing to the addition of antibodies against the autocrine receptor was demonstrated *(23)*. In the present study, we sought to quantify cellular response to IGF-1 and to determine whether a similar short-term exposure would lead to a measurable downstream cellular response. Exposure of the cells to IGF-1 did lead to dose-related changes in ECAR. This response is specific for IGF-1 and was inhibited by IGFBP-3. More importantly, this transient exposure can be correlated to proliferation, indicating the physiologic relevance of this signal. Our work validates this technique for the study of IGF-1 stimulation and suggests a new method for screening potential IGF-1 inhibitors.

Results

Optimization of Parameters for MAC-T Cell Line on Microphysiometer

The MAC-T cell line has not been examined previously using the Cytosensor Microphysiometer System, and, hence, optimization of conditions for evaluation of growth factor stimulation was needed. Optimization of this system included testing of seeding densities of MAC-T cells, IGF-1 challenge concentrations, IGF-1 challenge duration, and the number of challenge repetitions to which the cells would maximally respond. Optimized parameters for this cell line included a cell seeding density of 4.5×10^5 cells/transwell (5×10^5 cells/mL) followed by serum starvation for 72 h. Percentage ECAR (% ECAR) peak values were obtained within 14 min, as shown for IGF-1 at 5 ng/mL (Fig. 1). There was some variation in the peak value obtained between experiments performed on different days with, e.g., 10 ng/mL of IGF-1 resulting in peak %ECAR values between 110 and 116. Single challenges were used for analysis because repeated challenges with IGF-1 lead to a reduced %ECAR response, indicative of some type of downregulation within the stimulation pathway (Table 1). However, the desensitivity is not strictly owing to receptor downregulation, because a 14-min exposure to IGF-1 did not significantly reduce the availability of receptor binding sites (Table 2).

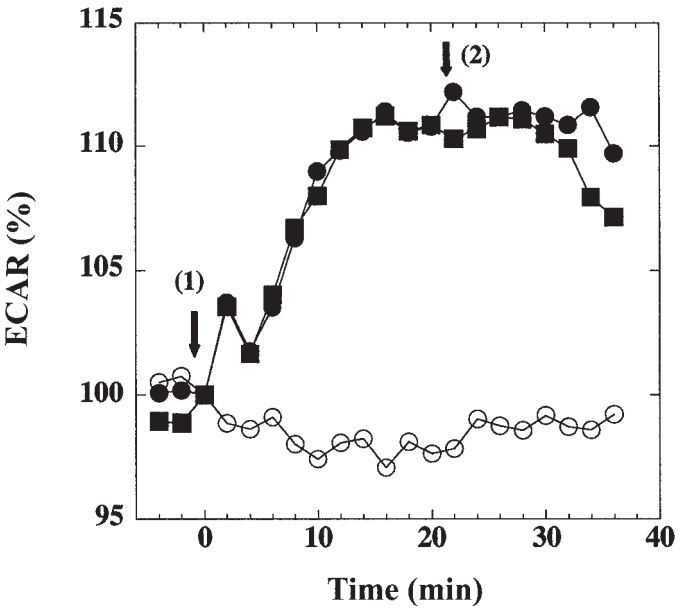


Fig. 1. Representative plot of microphysiometer measured response by MAC-T cells to 5 ng/mL of IGF-1. Transwells were seeded at 5×10^5 cells/mL and equilibrated for 1 h in running buffer prior to stimulation. Tracings for three channels are shown. Channels received IGF-1 at 5 (d , j) or 0 (s) ng/mL for 22 min followed by a switch to running buffer. Arrows indicate the valve switch to IGF-1 (1) and the return switch to control running buffer (2). Data are normalized to baseline at a point designated 0 min.

IGF-1 Concentration Dependency of ECAR Response by MAC-T Cells

We wished to determine whether this rapid ECAR response was concentration dependent and whether a maximal stimulation response occurred. The MAC-T response to varying concentrations of IGF-1 was determined by

Table 1
Decreased Response by MAC-T Cells with Repeated IGF-1 Stimulation[a]

IGF-1 challenge	IGF-1 (10 ng/mL)	IGF-1 (100 ng/mL)	IGF-1 (200 ng/mL)
1	100.0 ± 0.1	100.0 ± 0.3	100.0 ± 0.2
2	53 ± 5	40 ± 13	34 ± 12
3	56 ± 29		

[a]Results are reported as percentage of maximum response above normalized baseline for each concentration. Each result is the average of two or three different cell chambers from a single experiment. Similar results were found with repeated experiment. The standard error is given.

Table 2
Effect of Short-Term Exposure to IGF-1 on IGF-1R

Calculated parameters	No preincubation of cells	Preincubation with IGF-1 (50 ng/mL)
Receptors (no./cell)	45,000 ± 9000	33,000 ± 6000
K_d (M)	$6.2 \pm 2.0 \times 10^{-10}$	$6.2 \pm 1.8 \times 10^{-10}$
K_N	0.0076 ± 0.0014	0.0086 ± 0.0010

[a]Values are calculated from binding studies (eight concentrations, three wells/concentration) for a well exposed to 0 (control) or 50 ng/mL of IGF-1. Parameters were calculated with Mathematica® using the ligand-receptor binding model (equation 3). The standard error is given.

exposing cells to IGF-1 for 14 min at concentrations ranging from 0 to 200 ng/mL (Fig. 2). Minor day-to-day variation was noted with cells from different experiments; however, consistent dose-dependent responses were noted in all individual experiments. There was an increase in ECAR in response to increasing concentrations of IGF-1, with a maximal ECAR response resulting from exposure to 100 ng/mL of IGF-1. Note, however, that concentrations higher than 200 ng/mL were not examined because higher concentrations would fall outside the physiologic range.

Stimulation by IGF-1 Is Specific

Because IGF-1 stimulation has not been studied previously using microphysiometry, specificity of response was investigated. Insulin at concentrations <100 ng/mL is unable to displace bound IGF-1 from MAC-T *(17)*, and only at high concentrations (1000 ng/mL) does insulin stimulate these cells to proliferate, likely via cross-reactivity with IGF-1R *(24)*. Similar response profiles are found with short-term exposure to insulin using the microphysiometer (Fig. 3). Insulin at 10 ng/mL gave a lower response than IGF-1 at 10 ng/mL and was not significantly different from control ($p < 0.05$). In agreement with previous studies examining cell proliferation following long-term exposure (72 h) to insulin, high concentrations (1000 ng/mL) did lead to increased ECAR during the 14-min challenge even beyond that of 10 ng/mL of IGF-1. Transient exposure to IGFBP-3 (300 ng/mL) in the absence of

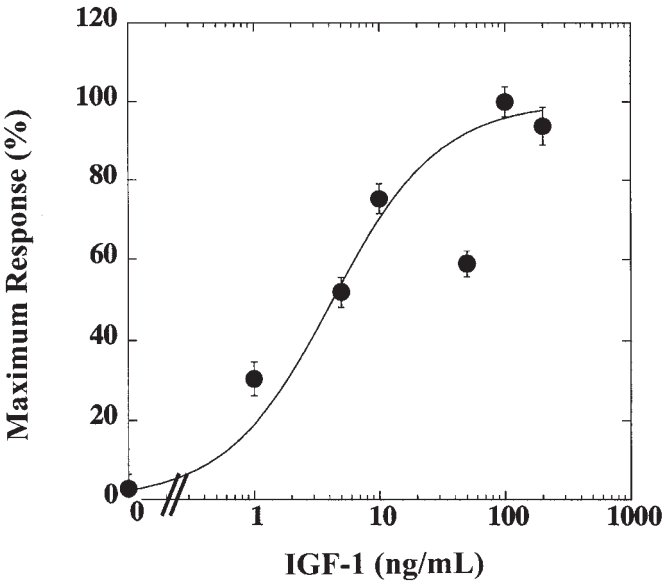


Fig. 2. Effect of IGF-1 on microphysiometer measured MAC-T response. Transwells were seeded at 5×10^5 cells/mL and exposed to IGF-1 concentrations from 0 to 200 ng/mL for 14 min. Peak %ECAR responses were seen with 100 ng/mL. The percentage of maximum response for each concentration was determined as the percentage of the 100 ng/mL response. Each point represents the mean ± SEM for at least two data points and at least two separate experiments.

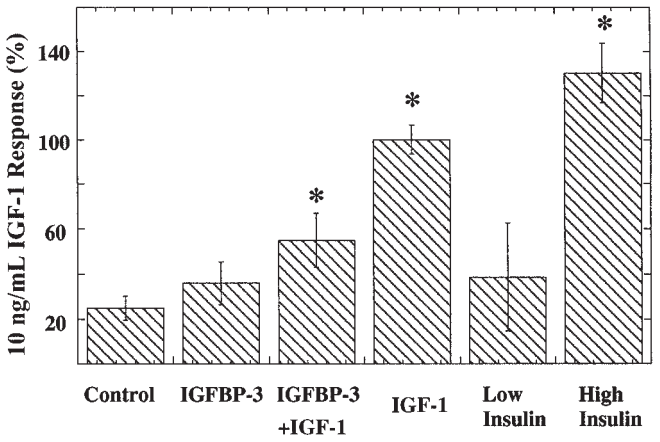


Fig. 3. Effect of insulin and IGFBP-3 on microphysiometer measured MAC-T response. Transwells were seeded at 5×10^5 cells/mL and exposed to IGF-1 (10 ng/mL), insulin (low, 10 ng/mL; high, 1 μg/mL) or IGFBP-3 (300 ng/mL) ± IGF-1 (10 ng/mL) for 14 min. The response is reported as the percentage of the 10 ng/mL IGF-1 response. Bars represent means ± SEM for at least two data points. *Significant difference from control (no additive) ($p < 0.05$).

IGF-1 was not significantly different from control ($p < 0.05$), confirming that specific cellular interaction is required for ECAR response and in agreement with our previous study showing that long-term exposure (16 h) to IGFBP-3 did not stimulate proliferation of MAC-T cells *(25)*. Simultaneous exposure to IGF-1 (10 ng/mL) and IGFBP-3 (300 ng/mL) did lead to a significant reduction in ECAR compared to IGF-1 alone ($p < 0.05$)—further evidence that changes in ECAR relate to specific cell association of the IGF-1.

Growth Response Resulting from Short-Term Exposure to IGF-1

To determine whether the ECAR signaling owing to transient exposure to IGF-1 would correlate with downstream cell activity, [^{3}H]thymidine incorporation studies were conducted. Exposure to IGF-1 for 14 min led to a significant ($p < 0.05$) dose-dependent increase in [^{3}H]thymidine incorporation (Fig. 4A). Even at a concentration as low as 10 ng/mL of IGF-1, [^{3}H]thymidine incorporation was significantly greater ($p < 0.05$) than for the control. This is not to suggest that longer exposure time does not have an effect on proliferation, since incorporation was significantly higher with 16 h of exposure (Fig. 4A). However, the overall response profiles for the 16 h and 14 min IGF-1 exposure are similar, with both reaching a plateau at 100 ng/mL (Fig. 4B). Furthermore, comparison of the IGF-1 concentration dependence, assayed using either microphysiometry or [^{3}H]thymidine incorporation, yields half-maximal effective concentrations (EC_{50}) that differ by less than an order of magnitude (Fig. 5).

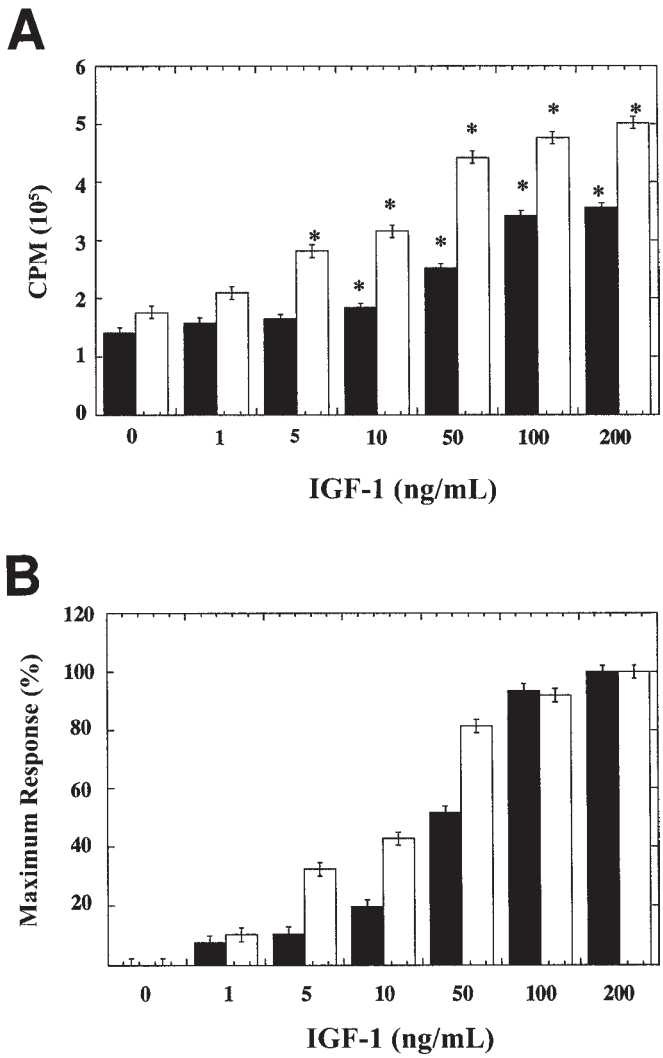
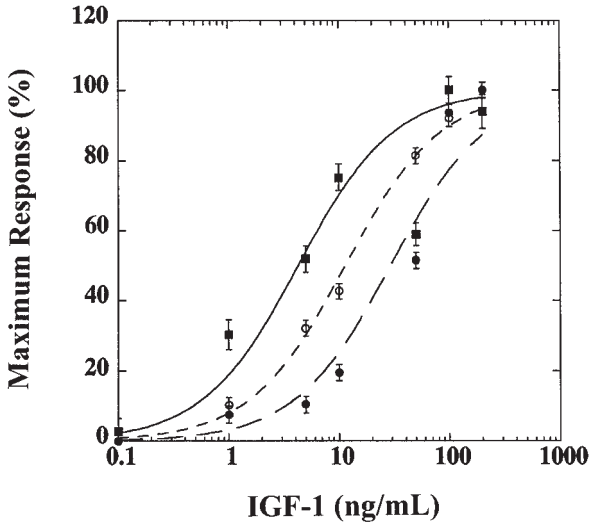


Fig. 4. Mean [^{3}H]thymidine incorporation by MAC-T cells in response to IGF-1. MAC-T cells were cultured in 24-well plates in the presence of IGF-1 for 16 h (h) or 14 min (j), followed by fresh serum-free media for the remaining 15 h and 46 min. A 2-h pulse with 1 μCi of [^{3}H]thymidine followed. **(A)** Direct cycles per minute for [^{3}H]thymidine incorporation is shown. Bars represent means ± SEM from four wells at each concentration. *Significant difference from control (0 ng/mL of IGF-1) ($p < 0.05$). Significance was determined separately for the 14-min and the 16-h treatment data sets. **(B)** Response shown is the percentage of the maximum response (100 ng/mL of IGF-1). Bars represent mean ± SEM.

Discussion

Regardless of whether IGF-1 is provided to mammary tissue via the circulation or via local tissue cells, the stimulation of cell proliferation influences mammogenesis. Our data serve to support the use of microphysiometry to study the effects of IGF-1 in bovine mammary epithelial cells and to provide evidence for physiologic relevance for the short-term response experimentally observed. Use of the microphysiometer with the MAC-T cell line offers a reductionist biologic model to study the effects and regulation of IGF-1 on mammary cells. Namely, it offers a means to

A



B

EC_{50} Response Values (ng/mL)

ECAR (%)	Thymidine Incorporation	
	14 min	16 h
4.3 ± 1.7	24.5 ± 8.1	9.0 ± 1.6

Fig. 5. Comparison of response measurements for the microphysiometer and [^{3}H]thymidine incorporation by MAC-T cells. **(A)** Response shown as percentage of the maximum response (100 ng/mL of IGF-1) for percentage of ECAR (j) and [^{3}H]thymidine at 16-h (s) and 14-min exposure (d). Data points represent the means ± SEM. Curves were obtained using least squares analysis of the data based on a saturation model. **(B)** EC_{50} ± SE was determined from the saturation model fit of the response profiles shown in (A).

measure responses in real time of a single clonal cell line in defined media. Although the use of isolated cell lines sacrifices the feedback mechanisms involved in tissue architecture, it significantly simplifies the system and allows less ambiguous interpretation of results. Contributions of IGFBP interaction and other factors in IGF-1 inhibition may also be more readily studied.

The conditions used for our studies were optimized for the MAC-T cell line, however, note that extension of this technology to other systems would require individual optimization. For example, Oehrtman et al. *(26)* indicated an optimal seeding of 2.5×10^5 cells/transwell with no serum starvation for investigating epidermal growth factor (EGF) stimulation of B82 mouse L-cells transfected to express human EGF receptor. Chan et al. *(21)* seeded mammary carcinoma cells at 3×10^5 cells/transwell and serum starved the cells for 24 h before testing with heregulin. Serum starvation synchronizes MAC-T cells *(27)* and was valuable for linking our short-term studies with cell proliferation. The prevalence of growth factors in serum, which can lead to receptor downregulation, also makes serum starvation a helpful technique for increasing signal generation. It is, however, not essential to the technique. The time required for peak response to IGF-1 on the microphysiometer (Fig. 1) is in agreement with other tyrosine kinase receptors *(19,21,26)* and is slow in comparison with G-coupled receptors *(28,29)*. This alone, however, does not illuminate which intracellular pathways are involved in the stimulation. Certainly the essentially immediate rise in %ECAR following addition of IGF-1 is similar to what has been seen using immunoprecipitation and Western blotting for phosphorylation of IGF-1R *(30)* and insulin receptor substrate-1 (IRS-1) *(31)* and IRS-2 *(32)*, early phosphorylation events. Peak phosphorylation of downstream targets such as Shc *(32)* or mitogen-activated protein kinase *(33,34)*, assayed again using immunoprecipitation and Western blotting, peaked at times on the order of 10 min, more in line with what we see regarding peak %ECAR. Our studies, however, have focused simply on the question of whether short-term exposure to IGF-1 can lead to downstream signaling. The addition of pharmacologic agents that interfere with specific pathways would be needed to decipher which steps within the stimulation pathway are involved in the process *(22,35)*.

We found the signal response by cells on the microphysiometer to IGF-1 to be rapid (Fig. 1) and concentration dependent (Fig. 2). Peak stimulation was seen with 100 ng/mL as the addition of 200 ng/mL of IGF-1 did not lead to significant additional stimulus (Fig. 2). This does not necessarily imply a saturation of IGF-1R, as receptor levels are not significantly reduced (Table 2), but a desensitization of some critical step in the extracellular acidification pathway may be occurring. To determine whether this short-term exposure to IGF-1 corresponded with cellular response, companion studies of [^{3}H]thymidine incorporation following IGF-1 addition were conducted (Fig. 4A). IGF-1 concentrations of 5 ng/mL or greater significantly ($p < 0.05$) stimulated DNA synthesis. Longer exposure times (16 h) did lead to greater incorporation of [^{3}H]thymidine, indicating that cellular signaling can occur beyond ECAR saturation (Fig. 4A). However, response profiles for maximal stimulation within each system were similar (Fig. 5), leading to EC_{50} values within the same range. These data strongly support our conclusion that changes in extracellular acidification in response to IGF-1 are physiologically relevant although they do not necessarily correspond directly with cell proliferation.

Although maximal responses to the addition of IGF-1 occurred on the microphysiometer approx 14 min following the addition of the growth factor to the environment, responses to IGF-1 were generally evident within the first 2 min cycle of IGF-1 exposure (Fig. 1). This result confirms that the microphysiometer can detect rapid real-time stimulation by IGF-1. IGF-1 has not been investigated previously using microphysiometry, although several other growth factors have been examined *(20,22,26,35–37)*. We confirmed the specificity of the MAC-T cell response to IGF-1 by comparing the response with insulin (Fig. 3). Our findings indicate a lack of response to a low physiologic concentration of insulin, which is in agreement with our earlier work indicating a lack of proliferative response to insulin at low concentrations *(24)*. The addition of insulin at higher concentrations known to displace IGF-1 from IGF-1R *(17)* and to stimulate cell proliferation *(24)* similarly induced an acute change in ECAR (Fig. 3).

The development of novel IGF-1 regulators may be facilitated by investigations using microphysiometry. For example, IGFBP-3 is known to bind with high affinity to IGF-1 in culture *(38)* and has been shown to affect IGF-1 stimulation of MAC-T cells *(25)*. In addition, IGFBP-3 is currently being investigated as an IGF-1-independent tissue regulator *(39)*, particularly of apoptosis *(40)*. Our studies (Fig. 3) are in agreement with our previous work *(24)* showing that IGFBP-3 affects MAC-T stimulation via an IGF-1-dependent rather than IGF-1-independent activity. This by no means implies that IGFBP-3 can not act independently in other cell types but indicates that the mechanisms by which this may occur are absent in MAC-T under our assay conditions. Furthermore, it illustrates how microphysiometry can be used to investigate IGF-1 inhibitors, particularly under clearance conditions, and may allow a distinction to be made between competitive and noncompetitive inhibitors.

Our study shows that short-term exposure to IGF-1 correlates with cell proliferation, an important downstream activity. We demonstrate that microphysiometry is a useful technique for investigating IGF-1 stimulation of mammary epithelial cells and that this tool has the potential to assist in rapid initial screenings for potential IGF-1 inhibitors or to assist in deciphering regulatory pathways when used in conjunction with traditional assays. Furthermore, it suggests that microphysiometry may be a useful tool for studying cell-mediated response for other mammary hormones both alone and coupled with IGF-1.

Materials and Methods

Cell Culture

The MAC-T cell line was maintained in complete media composed of Dulbecco's modified Eagle's medium (DMEM), 10 mL/L of Antibiotic-Antimycotic (100X), 1 mg/L gentamicin, 0.04 *M* $NaHCO_3$, and 10% fetal bovine serum (FBS). For cell studies, cells were plated at 1×10^6 cells/dish and grown to confluency, typically 48 h. Cells were then seeded at the designated concentration in transwells (microphysiometer studies) or 24-well plates ($[^3H]$thymidine incorporation studies and receptor binding studies). Cells were used between passages 6 and 16.

DMEM, Antibiotic-Antimycotic (100X), gentamicin sulfate solution (10 mg/mL), calcium-free Dulbecco's phosphate buffered saline (DPBS), trypsin (2.5% lyophilized), and FBS were purchased from Gibco (Grand Island, NY). Enzyme Free Cell Dissociation Solution was purchased from Specialty Media (Phillipsville, NJ).

Microphysiometer Experiments

Human recombinant IGF-1 and human recombinant IGFBP-3 were purchased from Upstate Biotechnology (Lake Placid, NY). Bovine insulin was from Lilly (Indianapolis, IN). Materials obtained from Molecular Devices for use on the microphysiometer included low-buffer Roswell Park Memorial Institute (RPMI) medium, capsule cup inserts, and spacer rings. Cells were seeded on tissue culture–treated transwells (polycarbonate membranes, 12 mm diameter, 3.0-μm pore size) (Corning Costar, Cambridge, MA). Transwells were seeded with MAC-T cells at 4.5×10^5 cells/transwell in complete media. Cells were incubated at 37°C for 24 h and the media replaced every 24 h with media without FBS for the next 72 h. RPMI (pH 7.4) supplemented with bovine serum albumin (BSA) (Fraction V; Sigma, St. Louis, MO) was used as the running buffer. Seeded transwell capsule cups were placed in the microphysiometer and equilibrated for approx 1 h in running buffer pumped at 100 μL/min. Cells were subsequently challenged with the protein of interest. Optimal results were obtained when each cell sample was challenged only once, and experiments for which maximal response was at least 10% above baseline were used.

Binding Experiments

MAC-T cells were plated at a density of 4.5×10^5 cells/well in 24-well plates and incubated in complete media for 24 h. Media was then changed to serum-free media, and spent media were replaced every 24 h. Cells were exposed to IGF-1 for 14 min whereas control cells were exposed to 14 min of fresh serum-free DMEM. Cells were washed with DPBS and incubated in binding buffer (0.5% BSA, 120 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgSO_4 \cdot 7H_2O$, 15 m*M* $NaC_2H_3O_2$, 100 n*M* HEPES, 10 m*M* dextrose) at 4°C for at least 20 min. Incubation with ^{125}I-IGF-1 followed for 3 h at 4°C. The cells were washed with DPBS, 0.3 *N* NaOH was added, and the plates were incubated overnight at 4°C. A COBRA™ II Auto-Gamma counter (Model D5002; Packard, Downers Grove, IL) was used for quantification.

Binding study results were interpreted using a simple one-site binding model *(41)*. We assumed each IGF-1R was able to bind with one IGF-1 and that there was no

receptor synthesis, degradation, or trafficking within our experimental system. Steady-state binding was assumed to occur within the 3-h experimental period, and no spatial dependence on concentration or ligand depletion was included. Nonspecific binding was assumed to be reversible and nonsaturable, and total bound IGF-1 reflected both specific and nonspecific binding. The equilibrium dissociation rate constant, K_d, the nonspecific association equilibrium constant, K_N, and the total IGF-1R sites per cell, were fit using a least-squares fit of data to a nonlinear parameter model using Mathematica (Wolfram, Champaign, IL). This analysis is mathematically equivalent to determining the nonspecific binding at a given ligand concentration based on linear extrapolation of the nonspecific bound in the presence of a saturating quantity of IGF-1 *(41)* with the advantage that this method utilizes the entire data set to determine the best fit of K_N, rather than relying on a single measurement.

[^{3}H]Thymidine Incorporation Experiments

[^{3}H]Thymidine uptake was measured as previously described *(15)*. Briefly, MAC-T cells were plated at 5×10^4 cells/well in 24-well plates in complete media. After 24 h, the cells were serum starved for 72 h. Cells were incubated with IGF-1 for 16 h or for 14 min, and the spent media was replaced with fresh media without FBS for the remaining incubation time. After 16 h, 100 μL of [^{3}H]thymidine (20 μCi/mL) (ICN, Irving, CA) was added to each well and incubation continued for 2 h. Wells were washed with DPBS followed by ice-cold 10% trichloroacetic acid and 100% ethanol washes. Plates were then incubated overnight with 0.3 *N* NaOH. Next, 3 *N* HCl was added to neutralize the NaOH, and samples from each well were quantified (Tri-Carb 2100 TR Liquid Scintillation Analyzer; Packard). Data were analyzed using the GLM procedure of SAS (SAS Institute).

Statistical Analyses

For [^{3}H]thymidine incorporation results, an analysis of variance was completed using the GLM procedure of SAS (SAS Institute). A Bonferroni (Dunn) *T*-test and specific contrasts were performed to determine significance of differences between treatments. For the microphysiometer studies, both the mean values and standard errors in Microsoft Excel (Delta Method for standard error) were calculated based on at least two replicates. Calculation of the EC_{50} based on the response profiles for microphysiometry and [^{3}H]thymidine were based on a saturation model equation and fit using KaleidaGraph (version 3.09, Synergy Software).

Acknowledgments

We appreciate the technical support and assistance by Lucy B. Gray in using the Cytosensor Microphysiometer and the assistance of Patricia L. Boyle in the cell culture preparation. This work was supported by a grant from The Whitaker Foundation.

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