Stimulation of DNA Synthesis and c-Fos Expression in Corneal Endothelium by Insulin or Insulin-like Growth Factor-I

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Purpose. To study the growth regulation of bovine corneal endothelium, the effects of insulin and insulin-like growth factor-I on the expression of the c-fos proto-oncogene as well as on DNA synthesis were analyzed. Bovine corneal endothelial cells were also analyzed for the presence of insulin and insulin-like growth factor-I receptors.

Methods. Indirect immunofluorescence was used to assess the effect of stimulation by insulin and insulin-like growth factor-I on c-fos protein expression and DNA synthesis quiescent bovine corneal endothelium. Receptor number was determined by 125I-insulin or 125I-IGF-I binding studies.

Results. Fetal bovine serum strongly stimulated c-fos protein expression and DNA synthesis. Insulin-like growth factor-I was less effective while insulin was effective only at high concentrations. Scatchard analysis of 125I-insulin-like growth factor-I and 125I-insulin binding to bovine corneal endothelium revealed 180,000 IGF-I receptors and 7,000 insulin receptors.

Conclusion. The number of insulin-like growth factor-I receptors far exceeds the number of insulin receptors in bovine corneal endothelium, suggesting that the effects of insulin on c-fos gene expression and mitogenesis were likely to be mediated through the insulin-like growth factor-I receptor. Invest Ophthalmol Vis Sci 1993;34:2105-2111.
Bovine corneas were dissected from eyes obtained from a slaughterhouse. In the authors’ opinion, the methods for securing bovine eyes were humane and complied with the ARVO Statement Use of Animals in Ophthalmic and Vision Research. The endothelial surface was treated with 0.05% collagenase (Sigma Corp, St. Louis, MO) for seventeen hours at 37°C. The solution was removed and plated in a 6 well dish (Falcon 3046, Lincoln Park, NJ) in F99 media (1:1 mixture of Ham’s F12 (UCSD Core Facility, La Jolla, CA) and Media 199 (UCSD Core Facility, La Jolla, CA)), supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics (penicillin 100 u/ml, streptomycin 100 μg/ml, UCSD Core Facility, La Jolla, CA). The primary cultures were selectively trypsinized to establish a pure population of BCE cells as determined by morphologic criteria and phalloidin staining (Feldman, unpublished data). Bovine corneal endothelial cells (passage 3–5) were plated onto glass coverslips at a density of approximately 80% confluency for immunofluorescent studies.

**MATERIALS AND METHODS**

**Cell Culture**

Bovine corneas were dissected from eyes obtained from a slaughterhouse. In the authors’ opinion, the methods for securing bovine eyes were humane and complied with the ARVO Statement Use of Animals in Ophthalmic and Vision Research. The endothelial surface was treated with 0.05% collagenase (Sigma Corp, St. Louis, MO) for seventeen hours at 37°C. The solution was removed and plated in a 6 well dish (Falcon 3046, Lincoln Park, NJ) in F99 media (1:1 mixture of Ham’s F12 (UCSD Core Facility, La Jolla, CA) and Media 199 (UCSD Core Facility, La Jolla, CA)), supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics (penicillin 100 u/ml, streptomycin 100 μg/ml, UCSD Core Facility, La Jolla, CA). The primary cultures were selectively trypsinized to establish a pure population of BCE cells as determined by morphologic criteria and phalloidin staining (Feldman, unpublished data). Bovine corneal endothelial cells (passage 3–5) were plated onto glass coverslips at a density of approximately 80% confluency for immunofluorescent studies.

**Immunofluorescence**

**Fos Expression.** Cells were made quiescent by replacing the culture media with that containing low amounts of FCS, 0.5%, for 24–48 hr. Various concentrations (10, 50, 100 ng/ml, 1,5,10 μg/ml) of insulin (Sigma Corp, St. Louis, MO) and IGF-I (Amersham International, Arlington Heights, IL) or 20% FCS were added. After 2 hr of stimulation, the cells were fixed with 3.7% formaldehyde and the membranes were made permeable with 0.3% Triton X-100 in phosphate buffered solution. The primary antibody, anti-fos (Oncogene Sci, Manhasset, NY; 1:30 dilution) was used for visualization (1:100 dilution, 30 minutes at 37°C). A biotinylated anti-rabbit antibody (Vector Labs, Burlingame, CA; 1:400 dilution, 1 hr at 37°C) was used to detect the anti-fos antibody. The fluorochrome, streptavidin Texas red (Vector Labs, Burlingame, CA) was used for visualization (1:100 dilution, 30 minutes at 37°C). Between all steps, coverslips were washed with phosphate buffered solution containing 0.05% nonidet p-40. The coverslips were then rinsed with water and mounted with gelvatol onto microscope slides for viewing.

**DNA Synthesis**

Quiescent cells were stimulated for 24 hrs with various concentrations of insulin or IGF-I, or serum as described earlier. Simultaneously, bromodeoxyuridine (BrdU, Amersham International, Arlington Heights, IL, 1:1000 dilution) was also added to the media for 24 hr. The coverslips were fixed with methanol (–20°C). The procedure used for immunofluorescent staining was similar to that described for c-fos except that the primary antibody was mouse anti-BrDU (Amersham International, Arlington Heights, IL) and the second antibody was a biotinylated goat anti-mouse antibody (Vector Labs, Burlingame, CA; 1:400 dilution). The fluorochrome remained the same.

**Statistical Analysis**

For the Fos or DNA synthesis experiments, two coverslips were used for each concentration of insulin, IGF-I, serum, or control. Four fields were examined so that a total of approximately 150–200 cells were counted. Each experiment was repeated a minimum of three times. Statistical analysis for all of the experiments was
performed on Statview 512+ (Brainpower Inc., Calabasas, CA). Analysis of variance testing was used to determine differences in labeling indexes between the growth factors insulin, IGF-I or serum from control. A probability value less than 0.05 was considered significant.

**Receptor Binding Assays**

Insulin and IGF-I binding to whole cells was quantified as previously described using A-14 moniodinated 

\[ ^{125}\text{I}-\text{insulin} \text{ and } ^{125}\text{I}-\text{IGF-I}, \text{ (donated by Lilly, Indianapolis, IN).} \]

Bovine corneal endothelial cells were split and plated into 35-mm plastic dishes for IGF-I binding studies. One hundred mm plastic dishes were used for insulin binding studies because of low binding. When confluent, the cells were washed three times with binding buffer (NaCl 131.2 mM, KCl 4.7 mM, CaCl\(_2\) 2.5mM, MgSO\(_4\) 1.24 mM Na\(_2\)HPO\(_4\) 2.5mM, 0.05% (BSA), pH 7.6). 

\[ ^{125}\text{I}-\text{IGF-I}, 0.03 \text{ nM}, \text{ or } ^{125}\text{I}-\text{insulin}, 0.16 \text{ nM}, \text{ and various concentrations of unlabeled insulin or IGF-I and binding buffer were added.} \]

After 6 hr incubation at 12°C, the cells were washed with phosphate buffered solution solubilized in 0.2M NaOH, 0.0% sodium dodecyl sulfate, and counted in a gamma counter. The specific cell-associated radioactivity was calculated by subtracting the amount of 

\[ ^{125}\text{I}-\text{insulin} \text{ or } ^{125}\text{I}-\text{IGF-I} \text{ bound in the presence of excess unlabeled insulin (17}\mu\text{M}) \text{ or IGF-I (261 nM)} \text{. Each hormone concentration was done in triplicate. Cell number was determined using a hemocytometer. Receptor number was calculated from Scatchard analysis of these separate experiments.} \]

Regression analysis was performed to determine if a one-site model applied.

**Crosslinking**

\[ ^{125}\text{I}-\text{IGF-I}, 0.6nM, \text{ was allowed to bind to BCE cells in the presence and absence of excess insulin (17}\mu\text{M}) \text{ as described above. Before washing, disuccinimidyl suberate in dimethyl sulfoxide 0.1 mM was added and the cells were incubated on ice for 20 min.} \]

The reaction was quenched with 10 mM Tris Hydroxy Methyl Aminomethane Hydrochloride, 1mM disodium ethylenediamine triacetate. After solubilization, the cross-linked cell solution was analyzed by 7.5% and 12% reduced sodium dodecyl sulfate, polyacrylamide gel electrophoresis, then by autoradiography.

**RESULTS**

To determine whether insulin or IGF-I induced c-fos protein expression, coverslips containing quiescent BCE were stimulated with various concentrations of insulin or IGF-I for 2 hr. BCE made quiescent by serum deprivation were found not to express c-Fos. The percentage of cells that produced the c-fos protein (i.e., specific nuclear staining) was determined by indirect immunofluorescence. IGF-I significantly induced c-fos expression at concentrations of 50 and 100 ng/ml in 48.6 ± 9.5% and 58.5 ± 8.9% of the cells, respectively, as compared with levels observed in quiescent controls, 10.4 ± 5.4% (P < 0.05, P < 0.05 respectively). Expression in response to IGF-I was well below the levels induced by stimulation with 20% FCS, (81.1 ± 2.0%; P < 0.05 for all concentrations of IGF-I) (Fig. 1). Conversely, insulin significantly induced c-fos expression only at the higher concentrations, 1,5 and 10 

\[ \mu\text{g/ml} \text{ (Fig. 2). The percentage of cells that expressed P}_{<0.05} \text{ for 1, 5, and 10 \mu g/ml of insulin was 46.0 ± 16, 56.8 ± 8.7, and 57.8 ± 13.7%, respectively, and was similar to those levels induced by 50 and 100 ng/ml of IGF-I.} \]

To determine whether insulin or IGF-I stimulated DNA synthesis, quiescent BCE were stimulated with various concentrations of insulin, IGF-I or 20% FCS for 24 hours in media containing BrDU as a labelling reagent. The percent of cells undergoing DNA synthesis was determined by counting cells exhibiting bright nuclei when stained with anti-BrDU. As with the c-Fos induction, IGF-I at a concentration of 100 ng/ml induced DNA synthesis significantly higher than control levels (55.5 ± 6.8% versus 26.2 ± 1.9%) (P < 0.05) but well below the levels induced by serum (82.6 ± 4.4%; Fig. 3). High concentrations of insulin (100ng/ml) were necessary to significantly increase DNA synthesis above levels seen in quiescent cells (Fig. 4).

Because of the high concentrations of insulin required for the mitogenic effect, binding studies were performed to determine the levels of IGF-I and insulin...
FIGURE 2. The percentage of cells that express Fos after stimulation of quiescent cells with 20% fetal calf serum, or insulin (0.01, 0.05, 0.1, 1, 5, and 10 μg/ml) for 2 hours. Each value represents the mean (± SEM) from three experiments. Significantly more cells expressed Fos at the high concentrations of insulin (1, 5, and 10 μg/ml) than uninduced quiescent cells or the lower concentrations of insulin 10, 50, and 100 ng/ml.

FIGURE 3. The percentage of quiescent BCE that underwent DNA synthesis after the addition of IGF-I (0.01, 0.05, 0.1 μg/ml) or serum (20%) for 24 hours. The addition of serum or IGF-I (100 ng/ml) significantly increased the proportion of cells that underwent DNA synthesis. Each value represents the mean (± SEM) of three experiments.

FIGURE 4. The percentage of quiescent BCE that underwent DNA synthesis 24 hours after the addition of insulin (0.01, 0.05, 0.10, 1, 5, and 10 μg/ml) or serum (20%). High concentrations of insulin (1.5, and 10 μg/ml) or serum significantly increased DNA synthesis. Each value represents the mean (± SEM) from three experiments.

receptors in these cells. Scatchard analysis of the 125I-IGF-I binding to BCE revealed approximately 180,000 IGF-I receptors per cell (Fig. 5a). A one-site linear model generated an r² value of 0.929. The EC50 of IGF-I binding was 0.6 nM (Fig. 5b). Scatchard analysis of 125I-insulin binding revealed that BCE express 7000 receptors per cell (Fig. 5c). The EC50 of insulin binding was 2.5 nM (Fig. 5d).

125I-IGF-I crosslinking studies were performed to confirm that the radioisotope was binding to the IGF-I and not the IGF-II receptor or IGF-I binding proteins (Figs. 6a, 6b). 125I-IGF-I bound to four bands. The band at 130 kD was most likely due to the presence of the alpha subunit of the IGF-I receptor, whereas the bands at 240 kD and the top of the autoradiograph likely represent alpha subunit oligomers. All three bands competed with insulin, suggesting that none represented the IGF-II receptor. Only a very faint band was seen at 40 kD (Fig. 6b), the size of IGF-I-binding proteins.

DISCUSSION

Insulin and IGF-I are two polypeptide growth factors that are closely related in amino acid sequence and may play a role in cellular regulatory mechanisms. Insulin is believed to be important in metabolism, whereas the IGFs appear to be involved in promoting growth. In this study, we demonstrate that insulin can induce DNA synthesis in BCE when used at high concentration, microgram rather than nanogram quantities. Conversely, IGF-I was able to induce DNA synthesis at nanogram concentrations. Woost et al have also recently shown that IGF-I was more effective than insulin at inducing DNA synthesis when measured by
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FIGURE 5. (a) Scatchard analysis of 125I-IGF-I binding to BCE. Receptor number was calculated using the x-axis intercept as fmol bound per 10^6 cells at 100% receptor occupancy. (b) This competition curve represents 125I-IGF-I. The EC50 is 0.6 nM. (c) Scatchard analysis of 125I-insulin binding to BCE. Receptor number was calculated using the x-intercept as fmol bound per 2.5 x 10^6 cell at 100% receptor occupancy. (d) The competition curve for 125I-insulin. The EC50 is 2.5 nM. These plots represent the mean data from three experiments, each performed in triplicate.

(total 3H-IdU5-125 iodo-2'- deoxyuridine) incorporation; a result confirmed by the present studies. However, because radioemulsion was not performed in the earlier studies, calculation of the percentage of cells undergoing DNA synthesis could not be determined.21 Because our studies use BrDU incorporation to determine the percentage of cells undergoing DNA synthesis, it is not possible to compare the percentage increases over control for these two independent studies. However, using two different means of measuring DNA synthesis in BCE cells, the results of the two studies agree that IGF-I is more effective than insulin.

We also investigated the effect of insulin and IGF-I on the expression of the protein product of the c-fos proto-oncogene. Induction of the c-fos gene is an early event after the addition of many growth factors, during proliferation and/or differentiation. The c-fos protein binds to the protein product of another proto-oncogene, c-jun and together, constitute transcription factor, AP-1.12 The effect of insulin and IGF-I on c-fos proto-oncogene expression has been examined in only a few cell types. In adipocytes and fibroblasts, insulin stimulates accumulation of c-fos messenger RNA22 whereas higher concentrations of IGF-I were necessary to observe this same effect. In H35 and H411EC3 hepatoma cells, insulin was also effective at inducing c-fos messenger RNA.23

We found that in BCE, the Fos protein was induced in response to both insulin and IGF-I. As in the case of DNA synthesis experiments, high concentrations of insulin were needed to stimulate significant levels of the c-fos protein. IGF-I significantly induced c-Fos expression at lower doses than necessary for its effect on DNA synthesis. In each case, levels of induction were not as high as serum, which might reflect that serum contains multiple growth factors.

Many cell types contain insulin and IGF-I receptors. Specific receptors exist at the cell membrane and...
are composed of at least two types of IGF receptors and one type of insulin receptor. Insulin binds with high affinity to the insulin receptor, low affinity to the Type 1 IGF receptor, and only poorly to the Type 2 IGF receptor. Conversely, IGF-I binds with the highest affinity to the Type 1 receptor and has a slightly lower affinity to the Type 2 IGF receptor. It binds very poorly to the insulin receptor. The insulin and Type I IGF receptors are similar: They are heterotetramers that exhibit autophosphorylation and tyrosine kinase activity. There is some evidence that the metabolic actions of insulin and the IGF are mediated through the insulin receptor and that the growth promoting effects of insulin and the IGF are mediated by the Type-I IGF receptor.

The binding and crosslinking studies performed on BCE presented here demonstrate that these cells possess 7,000 insulin receptors. These data agree with those pertaining to the induction of c-fos and DNA synthesis: insulin is only able to stimulate these cells at concentrations high enough to allow crossover binding into the IGF-I receptor. As approximately 180,000 IGF-I receptors are present on these cells, both the IGF-I and insulin stimulated DNA synthesis is most likely occurring by way of signal transduction processes originating with the IGF-I receptor. None of the IGF-I binding studies appeared to be caused by IGF-II receptors because insulin efficiently competed with IGF-I for binding to all the bands, and insulin does not bind to the IGF-II receptor. Thus, the bands probably represent DSS crosslinked oligomers of the IGF-I receptor. These crosslinking studies also show that less than 1% of the total IGF-I binding was bound to IGF-I binding proteins.

In summary, the dose dependent IGF-I stimulated c-fos and DNA synthesis demonstrated in BCE cells are likely attributable to binding to the IGF-I receptor. The c-fos protein and DNA synthesis stimulated only at high concentrations of insulin are also likely occurring via the IGF-I receptor, a receptor present on the surface of BCE in great abundance over the insulin receptor. Thus, the IGF-I receptor may represent a potential target for regulating the growth of corneal endothelial cells in culture or in vivo.

Key Words
insulin-like growth factor I (IGF-I), insulin, proto-oncogene expression, DNA synthesis, insulin receptor, IGF-I receptor

Acknowledgments
The authors thank Jerrold M. Olefsky, M.D. for his assistance and helpful discussions in these studies.

References
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