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Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts *in vitro*

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Summary

Previous studies showed that insulin promotes cell proliferation and morphological development of preimplantation mouse embryos. In this report, the receptor responsible for these actions and the cell populations that are affected were investigated. Insulin's 9 % stimulation of blastocyst cell number was entirely due to a 23 % increase in ICM cell number with an EC_{50} of 0.54 pM. This and the similar degrees of stimulation of immunosurgically isolated ICMs by both physiological and supraphysiological insulin concentrations suggest that insulin receptors are present on the ICM and respond to exogenous insulin transcytosed through the TE to promote expansion of the ICM cell numbers. In morphological studies, insulin increased the number of blastocysts and decreased the number of morulae by 10 % after 54 h culture from 2-cell embryos with EC_{50}s of about 0.95 pM. The equivalence of these EC_{50}s suggests mediation of insulin's stimulation of blastocyst formation *via* insulin receptors which are functionally expressed around the time of compaction at the 8-cell stage. These results support our hypothesis that insulin has an important role in the regulation of growth during preimplantation development.

Key words: insulin, insulin receptor, pre-embryo, mitogen.

Introduction

Although preimplantation embryos may develop normally in a chemically defined medium (Biggers *et al.* 1971), their cleavage rate is slower than of embryos developing *in vivo* (Bowman and MacLaren, 1970; Harlow and Quinn, 1982). This and the current interest in increasing the success of human *in vitro* fertilization (IVF) programs has prompted investigation of the effects of additives to the culture medium on embryo development. Protein supplements and numerous serum sources have been explored with varying success. The maternal system may produce specific factors to optimize embryo development *in vivo*.

Insulin is a requirement for growth of many cells in culture and stimulates amino-acid transport (Kaye *et al.* 1986), protein synthesis (Harvey and Kaye, 1988) and enhances cleavage and morphological development when added to the cultures of mouse embryos *in vitro* (Gardner and Kaye, 1990). In protein synthetic studies, insulin's effect was shown to be mediated *via* insulin receptors present on cells of both the trophectoderm (TE) and inner cell mass (ICM) of the blastocyst (Harvey and Kaye, 1990*a*).

The aim of the present study was to investigate the receptor responsible for insulin's promotion of expansion of blastocyst cell numbers and blastocyst formation by closely examining the concentrations at which insulin exerted its stimulation. Furthermore, the cell population that is affected by insulin was examined by differential nuclear staining of the TE and ICM.

Materials and methods

Superovulation and embryo manipulation

Randomly bred, Quackenbush mice (10 weeks) were superovulated by i.p. injection of 10 i.u. PMSG at 10.00 h followed 48 h later by 10 i.u. hCG (Folligon and Chorulon, Intervet, Aust) and paired with males. Mating was determined by the presence of a vaginal plug at 9.00 h the following morning. Two-cell embryos were collected 48 h post-hCG in M2 (Fulton and Whittingham, 1978; modified as previously described, Hobbs and Kaye, 1985) and cultured in 30 μl droplets of BMOC2 (Brinster, 1965; modified as previously described, Pemble and Kaye, 1986) under liquid paraffin oil at 37°C in a humidified atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2.

Insulin effect on cell number and morphological development

Two-cell embryos were cultured 54 h with various concentrations of insulin (Humulin R, Eli Lilly, Aust). The m-cresol and glycerol, contained in Neutral Humulin solution, did not affect development or protein synthetic rate of embryos during this period at appropriate concentrations (Harvey and

Kaye, 1988) and so control media contained no additives. Following culture, the number of cells in the TE and ICM were counted after differential nuclear staining (Handyside and Hunter, 1984). The zonae pellucidae were removed in acid Tyrode's solution (pH 2.5; Hogan *et al.* 1986) before incubation in anti-mouse serum (1:5 dilution in M2) for 10 min at 37°C. Embryos were then washed with M2 and incubated 5 min at 37°C with $10\,\mu g\,ml^{-1}$ propidium iodide (Sigma, St Louis, MO, USA) and a 1:10 dilution of normal guinea pig serum as a source of complement. They were briefly washed in 10 mM phosphate buffer (pH 7.2) containing 137 mM NaCl and 2.7 mM KCl before transfer to absolute ethanol with $25\,\mu g\,ml^{-1}$ bisbenzimide (Hoechst 33258; Sigma, St Louis, MO, USA) and left overnight at 4°C. The following morning they were washed with absolute ethanol before mounting in a hanging drop of glycerol and visualized under a Zeiss Photomicroscope 2 fitted with a HBO 50W high pressure mercury lamp. The TE nuclei were stained red using an excitation filter set comprising band pass interference exciter filter 546/12, barrier filter LP 590 and chromatic beam splitter FT 580 (Zeiss), whilst the nuclei of both cell populations were visible with blue–violet excitation (filter set comprising band pass interference exciter filter G 365, barrier filter LP 420 and chromatic beam splitter FT 395; Zeiss), the TE nuclei being pink and the ICM nuclei blue.

Prior to staining, embryos were classified as blastocysts, morulae or cleavage-stage embryos, which were those that had developed to the 8-cell stage but had not undergone compaction i.e. there were distinct blastomeres with no interblastomeric junctions visible.

Insulin effect on isolated ICM

The ICMs of blastocysts collected 96 h post-hCG were isolated (Solter and Knowles, 1975 as modified by Harvey and Kaye, 1990*a*) and cultured 24 h in various concentrations of insulin before determination of cell number using bisbenzimide (Hoechst 33258) as above. Briefly, the zona pellucidae were removed (as above) and blastocysts exposed to a 1:10 dilution of heat inactivated, rabbit anti-mouse serum in M2 at 37°C for 10 min. They were then washed free of unbound antiserum with M2 and transferred to a 1:10 dilution of normal guinea pig serum in M2 at 37°C; which acted as a source of complement resulting in lysis of the TE cells and exposed the ICMs. Previous experiments confirmed that ICMs were not contaminated with TE cells or debris.

Statistical analysis

Statistical analysis of cell number studies was by ANOVA. The percentages of 2-cell embryos developing to the various morphological stages after 54 h culture were transformed to radians and analysed by paired *t*-test.

Results

Effect of insulin on cell number

Blastocysts that had developed with 1.7 nM insulin contained 9 % more cells than those in control medium. This increase was entirely due to a 23 % increase in the mean cell number of the ICMs. Insulin had no effect on trophectoderm proliferation (Fig. 1). The frequency distribution for the ICMs shows no evidence of insulin unresponsive embryos (Fig. 2). In dose–response studies, there was no effect at 0.17 pM but a significant correlation ($P<0.01$, $r=0.9962$, $n=4$) between the log of the insulin concentration and ICM cell number in the range 0.17–1.7 pM insulin ($EC_{50}=0.54$ pM) when these data were fitted by least squares linear regression (Fig. 3).

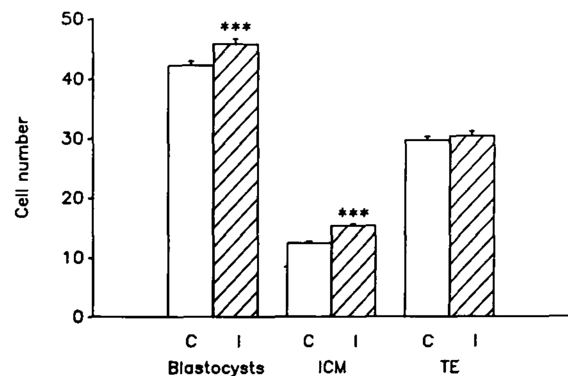

Fig. 1. Cell number of whole blastocysts, inner cell mass (ICM), and trophectoderm (TE) after 54 h culture in BMOC2 (C) or BMOC2+1.7 nM insulin (I). Means±S.E.M. of 3 experiments each containing at least 50 blastocysts/treatment. ***, $P<0.001$.

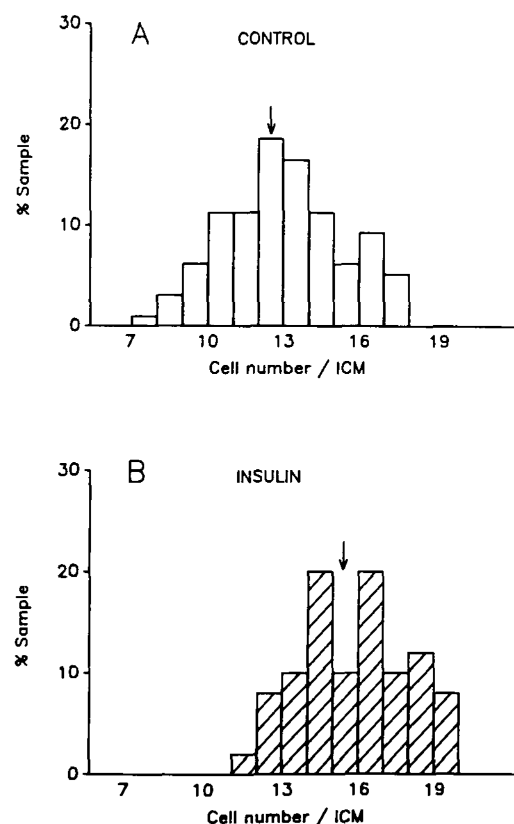

Fig. 2. Frequency distribution of ICM cell number in blastocysts from BMOC2 (control) medium and BMOC2+1.7 nM insulin. Means indicated by arrows. Data from Fig. 1.

Insulin's effect on isolated ICMs

Both 1.7 pM and 1.7 nM insulin stimulated ICM cell numbers by 25 % when they were collected from 96 h post-hCG blastocysts and cultured 24 h (Fig. 4).

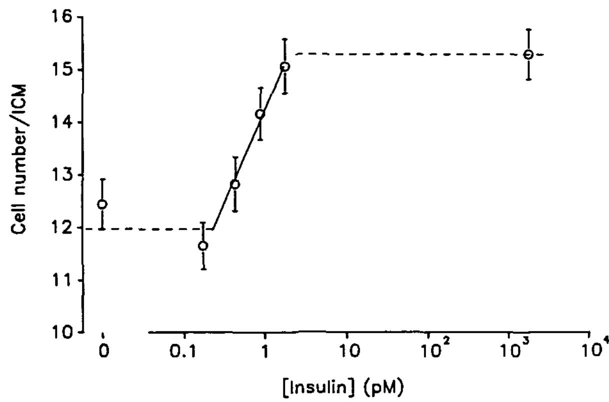

Fig. 3. Cell number of ICMs of blastocysts cultured 54 h from 2-cell embryos in BMOC2 media containing various concentrations of insulin. Points are means±S.E.M. of 3 experiments each containing 26 ICMs/treatment. Full line fitted by least squares linear regression.

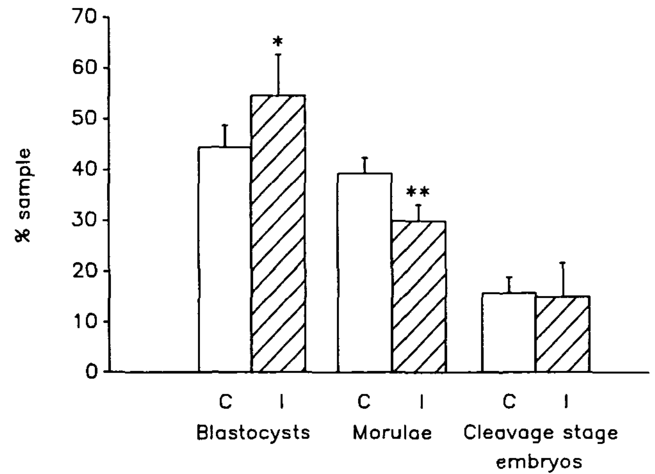

Fig. 5. Percentage of embryos reaching the various morphological stages after 54 h culture from 2-cell embryos in BMOC2 (C) and BMOC2+17 pM insulin (I). Means±S.E.M. of 6 experiments with at least 25 embryos/experiment. *, $P<0.05$; **, $P<0.01$.

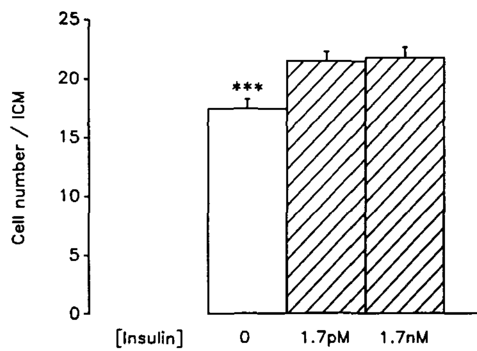

Fig. 4. Cell number of ICMs isolated from blastocysts (collected 96 h post-hCG) and cultured 24 h in BMOC2+0, 1.7 pM, and 1.7 nM insulin. Means±S.E.M. of 4 experiments each containing at least 21 ICM/treatment. ***, $P<0.001$.

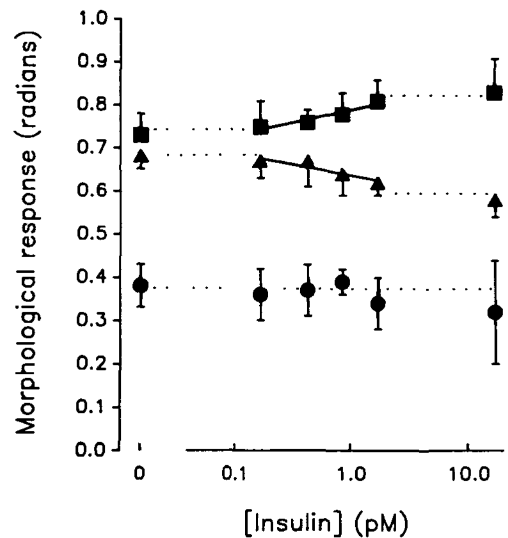

Fig. 6. Dose–response curves of the percentage of 2-cell embryos reaching morphological stages (converted to radians) to insulin after 54 h culture. Points are means±S.E.M. of 3 experiments with at least 25 embryos/experiment. Squares, blastocyst; triangles, morulae; circles, embryos remaining in cleavage stages. Full lines fitted by least squares linear regression.

Effect of insulin on morphological development

After 54 h culture, 10.2 % more blastocysts and 9.4 % fewer morulae were found in the presence of 17 pM insulin (Fig. 5). Insulin had no effect on the numbers of cleavage-stage embryos observed at this time. In dose–response studies, 0.17 pM insulin gave no response, but there was significant correlation between the insulin concentration and the proportion of blastocysts ($P<0.01$, $r=0.9957$, $n=4$) and morulae ($P<0.05$, $r=-0.9832$, $n=4$) in the range 0.17 pM to 1.7 pM when these data were fitted by least squares linear regression (Fig. 6). The EC_{50}s were indistinguishable at 0.90 pM for blastocysts and 0.97 pM insulin for morulae.

Discussion

Previous work showed that addition of insulin to the culture medium of 2-cell embryos developing to blastocysts increased cell numbers and rate of morphological development (Gardner and Kaye, 1990). This increase in cell number may arise from mitogenic action or by decelerating cell death and so promoting cell survival. Thymidine incorporation studies (Heyner *et al.* 1989) and increased mitotic index coincident with increased cell number of embryos cultured with insulin (Gardner and Kaye, 1990) suggest that insulin was mitogenic in its stimulation of embryonic cell number. Furthermore, only about 4 % of cells were found to be

dead in blastocysts with approximately the same cell numbers (Copp, 1978). Since this represents loss of less than one inner cell whilst insulin caused a 23 % increase (3 cells); it is most likely that insulin was acting as a mitogen, although it may also have reduced cell death. The present experiments were designed to identify the receptor responsible for insulin's growth effects and determine in which of the blastocyst tissues the increased numbers of cells were located.

The increase in the total cell number of blastocysts was entirely due to proliferation of the ICMs (Fig. 1), which all appeared to be responsive to insulin (Fig. 2). Day 19 fetuses derived from blastocysts transferred after 48 h culture with insulin from the 2-cell stage, showed a 10 % increase in fetal weight and no effect on placental weight (Gardner and Kaye, 1986). Since the ICM is the anlagen for the fetus proper, the present observations indicate a direct link between the increased number of ICM cells induced by insulin during the preimplantation period and this increase in fetal weight. Furthermore, insulin had no effect on the TE (from which the embryonic components of the placenta arise) correlating with no effects of insulin on placental weight.

The EC_{50} of 0.54 pM insulin for stimulating ICM cell number in whole blastocysts (Fig. 3) is identical to that for stimulation of embryonic protein synthesis which has been confirmed to be *via* insulin and not IGF-1 receptors (Harvey and Kaye, 1990*a*). The specific binding of ^{125}I-insulin on mouse blastocysts at concentrations less than 1 pM (Mattson *et al.* 1988), together with the very low EC_{50} for insulin's stimulation of metabolism (Harvey and Kaye, 1988), ICM cell numbers and blastocyst formation reported here, strongly support the hypothesis that insulin receptors mediate these diverse developmental effects of insulin.

Although, insulin may act *via* IGF-1 receptors in various cells, it mediates cellular proliferation *via* its own receptor in embryonal carcinoma and neuroblastoma cells (Nagarajan and Anderson, 1982; Mattsson *et al.* 1990). Furthermore, insulin's stimulation of mitosis in hepatoma cells has been attributed to mediation *via* the insulin receptor on the basis of an EC_{50} of 30–70 pM (Koontz and Iwashashi, 1981) and use of anti-receptor antisera (Koontz, 1984; Mottola and Czech, 1984). Therefore, mouse blastocyst insulin receptors mediate insulin's actions at concentrations 100-fold less than those required to activate receptors on classical insulin target tissues. This high-sensitivity may protect the embryo from feeding-related fluctuations in maternal insulin levels.

The TE epithelium completely envelopes the ICM, and so must bind and transcytose insulin at these concentrations to affect the ICM. The above results do not indicate the sensitivity of insulin receptors on the ICM, or exclude the possibility that upon binding to the TE, insulin may stimulate the production of another growth factor to be released to stimulate the ICM. The identical increases in ICM cell numbers of immunosurgically isolated ICMs with both concentrations of insulin (Fig. 4) indicate that insulin receptors on the ICM also mediate insulin's effect on this tissue and suggest that a paracrine system involving another factor is unlikely. Thus, insulin binds to insulin receptors on the TE (maximal response at 1.7 pM insulin; Fig. 3), is transcytosed across the epithelium to bind to insulin receptors on the ICM (maximal response at 1.7 pM insulin; Fig. 4) and stimulates mitogenesis. Similar effects were seen with insulin's stimulation of protein synthesis *via* insulin receptors on the ICM in intact blastocysts (Harvey and Kaye, 1990*a*), but in the case of short-term stimulation of protein synthesis the TE cells were also stimulated. These conclusions support the electron microscopic visualisation of gold-labelled insulin in the TE, between the membranes of the TE and ICM, and binding to the ICM in mouse blastocysts (Heyner *et al.* 1989).

Epidermal growth factor (EGF) has been shown to affect the TE only with no effect on the ICM (Wood and Kaye, 1989). Thus different growth factors affect different cell populations of the blastocyst and there exist specific transport mechanisms permitting transcytosis of selective ligands through the TE to affect the ICM. Furthermore, insulin has selective effects on the different cell populations of the blastocysts, stimulating protein synthesis in both the ICM and the TE (Harvey and Kaye, 1990*a*) whilst stimulating the number of cells in only the ICM.

In confirmation of other studies (Gardner and Kaye, 1990), insulin enhanced blastocyst formation resulting in a decreased number of morulae after 54 h culture from 2-cell embryos (Fig. 5). The EC_{50}s of about 0.95 pM insulin (Fig. 6) indicate that the same high sensitivity insulin receptor mediates both stimulation of cell number and morphogenesis.

The absence of any effect on the number of early cleavage stage embryos after 54 h (Fig. 5) suggests that the insulin receptors appear around the time of compaction and not before. This appearance coincides with observations of specific insulin binding to morulae and blastocysts but not to earlier embryonic stages (Mattson *et al.* 1988), visualization of insulin receptors on compacting/compacted embryos and not earlier (Harvey and Kaye, 1990*b*), and insulin's stimulation of protein synthesis in compacted but not in uncompacted eight- cell embryos (Harvey and Kaye, 1988). Furthermore, insulin receptor mRNA has been detected around the 8-cell stage and in later developed embryos but not earlier (Rappolee *et al.* 1989).

Immunohistochemical studies have shown that insulin internalized by freshly collected embryos is maternally derived (Heyner *et al.* 1989). Moreover, in diabetic mice, blastocysts have fewer cells and lower protein synthetic rates than in normal mice (Beebe and Kaye, 1990). Fewer ICM cells were also observed in blastocysts from diabetic rats (Pampfer *et al.* 1990). It is therefore likely that these stimulatory effects of insulin observed *in vitro* also apply *in vivo* and that the source of insulin to the developing embryo is maternal.

In conclusion, insulin enhances embryonic development and is specifically transcytosed from the external environment through the TE to stimulate mitosis of the

ICM. These effects are mediated *via* receptors which are sensitive to extremely low insulin concentrations suggesting a mechanism by which the embryo may be continually responsive to basal maternal insulin levels.

Furthermore, the selective enhancement of blastocyst formation and stimulation of cleavage in the newly differentiated ICM indicate that insulin may promote differentiation and proliferation of this tissue, from which the fetal tissues are derived. Thus, insulin appears to be a specialized early embryonic growth factor; a role that may have evolved prior to its more common endocrine function, because similar activities are seen in the regulation of growth and differentiation in early chicken embryos (De Pablo *et al.* 1985), stimulation of RNA synthesis in sea urchin embryos (De Pablo *et al.* 1988), and reinitiation of meiosis in amphibia and goldfish oocytes (Lessman and Marshall, 1984; Lessman, 1985).

The absence of insulin and other growth regulatory factors from the culture medium may explain the retarded cleavage seen in embryos developing *in vitro* (Bowman and MacLaren, 1970; Harlow and Quinn, 1982).

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