Stimulation of endocytosis in mouse blastocysts by insulin: a quantitative morphological analysis

G. F. Dunglison^{1,2*}, S. D. Jane³, T. F. McCaul⁴, J. E. Chad³,
T. P. Fleming² and P. L. Kaye^{1†}

¹Department of Physiology and Pharmacology, The University of Queensland, Brisbane, Queensland 4072, Australia; ²Department of Biology and ³Department of Physiology and Pharmacology, School of Biological Sciences, The University of Southampton, Southampton SO16 7PX, UK; and ⁴Centre for Microscopy and Microanalysis, The University of Queensland, Brisbane, Queensland 4072, Australia

The effects of insulin on the endocytic activity of mouse blastocysts *in vitro* were investigated using confocal laser scanning microscopy, quantitative image analysis and electron microscopy. Confocal studies showed that fluorescein isothiocyanate-labelled markers, dextran (fluid phase) and albumin (combined membrane and fluid phase), were endocytosed by blastocysts and localized within vesicles (about 2.5 μm in diameter) in the outer trophoctoderm cells. No labelling was detected in the inner cell mass cells or the blastocoel cavity. Treatment with 170 nmol insulin l^{-1} stimulated the endocytosis of fluorescently labelled dextran in freshly collected blastocysts, increasing mean vesicle diameter per embryo by 15% ($P < 0.05$) after incubation with insulin for 2.5 h and mean vesicle number per embryo by 56% ($P < 0.01$) after 6 h. Both effects were also evident in blastocysts that had been cultured from the late eight-cell stage. Blastocysts incubated for 6 h with insulin displayed increased convolutions in the trophoctoderm apical membrane compared with controls, indicating increased membrane activity and suggesting macropinosome formation. Collectively, these results suggest that insulin enhances endocytosis in the trophoctoderm by stimulating uptake at the apical membrane into larger and more numerous endocytic vesicles and with some evidence of vesicle fusion. This mechanism may provide a metabolic basis for the stimulation by insulin of biosynthesis, proliferation and morphological development in early embryos.

Introduction

After fertilization, mouse embryos develop to blastocysts of about 125–250 cells before implantation, late on day 4 of gestation. During this period, the developing embryo is free in the oviduct or uterus and must rely on endogenous stores or components of the fluids of the reproductive tract to meet the various requirements of cell proliferation and differentiation. Overt differentiation commences at the eight-cell stage, leading to the formation of two fundamental cell lineages of the blastocyst, the outer epithelial trophoctoderm and the inner cell mass (ICM; reviewed in Fleming, 1992). The fetus develops from the ICM, while the trophoctoderm cells give rise to non-fetal components (reviewed in Gardner, 1983).

Mouse embryos take up nutrients by different mechanisms, depending upon nutrient size and charge. Small solutes such as amino acids, glucose and pyruvate, use specific transport systems for their uptake (Kaye, 1986; Gardner and Leese, 1988; Van Winkle, 1988). However, large molecules such as proteins

are available to the embryo only by receptor-mediated endocytosis (Heyner *et al.*, 1989; Smith *et al.*, 1993), endocytosis of protein in the fluid phase or nonspecific adsorption to the cell membrane (Fleming and Pickering, 1985; Fleming and Goodall, 1986; Pemble and Kaye, 1986). The fluids of the reproductive tract are rich sources of protein (Gardner and Leese, 1990); the operation of the latter endocytic pathway may therefore be of great nutritional potential to the developing embryo.

Embryos developing *in vivo* endocytose BSA injected into the maternal circulation, suggesting that maternal serum proteins are taken up by nonspecific endocytosis (Glass, 1963). Using uptake of radioiodinated BSA *in vitro*, Pemble and Kaye (1986) observed a large increase in endocytic activity at blastocyst formation, reaching a rate of fluid uptake equivalent to 2.5% of the total blastocyst volume per minute. This surge in endocytic activity at the blastocyst stage coincides with the time at which the trophoctoderm endocytic apparatus matures and becomes spatially organized such that endocytosis occurs preferentially from the outer (apical) membrane (Fleming and Pickering, 1985; Fleming and Goodall, 1986). Thus, as the embryo differentiates, its endocytic system becomes a highly active conduit for maternally derived nutrient uptake to support early growth and development.

*Current address: Department of Human Morphology, Queen's Medical Centre, The University of Nottingham, Nottingham, NG7 2UH, UK.

†Correspondence.

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In many cell types, the activity of the endocytic system and of the plasma membrane may be influenced by hormones and growth factors, such as insulin (Brunk *et al.*, 1976; Haigler *et al.*, 1979; Kadowaki *et al.*, 1986; Miyata *et al.*, 1988). Blastocyst physiology responds to insulin with stimulation of protein synthesis, mitogenesis and morphological development (Harvey and Kaye, 1988, 1990; Heyner *et al.*, 1989; Rao *et al.*, 1990; Gardner and Kaye, 1991; Kaye *et al.*, 1992; Kaye and Harvey, in press) and in particular, an increased rate of endocytosis of radiolabelled BSA (Dunglison and Kaye, 1993) and dextran (Dunglison and Kaye, 1995).

In view of these findings, and to gain a better understanding of the mechanism of this increased activity, we have quantified the morphological effects of insulin on endocytosis in mouse blastocysts using confocal and electron microscopy.

Materials and Methods

Embryo collection

All experiments were approved by the Institutional Animal Ethics Committee as conforming to NHMRC guidelines. Female Quackenbush (Gore Hill Research Laboratory, University of Technology, Sydney; 4–5 weeks old) or MF1 mice (Olac-derived, Southampton University Animal House; 3–4 weeks old; used for fluorescein isothiocyanate (FITC) dextran labelling confocal studies) were superovulated by i.p. injection of 5–10 iu pregnant mares' serum gonadotrophin (Folligon, Intervet, North Ryde and Cambridge) at 10:00 h or 14:00 h, respectively, and 5–10 iu hCG (Chorulon, Intervet) 48 h later. The mice were then paired overnight with individual males of the same breed. The presence of a vaginal plug the next morning indicated mating. Embryos were recovered by flushing oviducts and uteri at the late eight-cell stage (72 h after hCG injection) or the mid-blastocyst stage (96 h after hCG injection) in medium 2 (M2; Fulton and Whittingham, 1978). Blastocysts used for FITC dextran studies were cultured in medium 16 (M16; Whittingham, 1971) under oil at 37°C and 5% CO₂ in air. Late eight-cell embryos were cultured under the same conditions for approximately 20 h until they had formed blastocysts. Blastocysts used for FITC albumin or ultrastructural studies were cultured in BMOC2 (Brinster, 1965; modified as described by Hobbs and Kaye, 1985) under oil at 37°C in an atmosphere of 5% CO₂, 5% O₂, 90% N₂. Unless noted otherwise, M2 and M16 both contained 4 g BSA l⁻¹; and BMOC2 contained 1 g BSA l⁻¹.

Fluorescence labelling

Freshly collected blastocysts (96 h after hCG injection; blastocoel cavity occupying approximately half of the blastocyst volume) were incubated in M16 containing 12.5 g FITC dextran l⁻¹ (9.4×10^3 M_r; Sigma Chemical Company, St Louis, MO) or BMOC2 containing 12.5 g FITC BSA l⁻¹ (67×10^3 M_r; Sigma Chemical Company) at 37°C or 4°C for 2 h, collected and washed four times in M2 at 25°C, before fixation in 2% (w/v) paraformaldehyde in PBS, pH 7.4 for 1 h. After collecting and washing four times in PBS, the blastocysts were transferred in a minimal volume of PBS to poly-L-lysine

coated coverslips within immunolabelling chambers. Coverslips were placed on top of the chambers to prevent the drying of embryos. Three control and three insulin-treated blastocysts were placed in the same immunolabelling chamber and visualized using confocal microscopy.

Embryos were incubated in M16 in the presence or absence of 170 nmol insulin l⁻¹ (Humulin R, Eli Lilly, Basingstoke) at 37°C for 20 h (eight-cell embryos) or 0.5, 2 and 4 h (blastocysts), transferred to the same medium containing FITC dextran at 37°C for 2 h labelling, and then processed for confocal studies as described above.

Confocal microscopy and image analysis

FITC-labelled embryos were visualized using a BioRad MRC 600 Series Confocal Imaging System equipped with an argon ion laser producing an excitation wavelength of 488 nm. This system permitted the imaging of individual optical planes of intact blastocysts without out-of-focus contamination. Confocal settings were standardized for insulin-treated and control embryos (confocal aperture set at 4; black level set at zero light). A series of optical sections (12 per embryo, each approximately 3 µm thick) was recorded at 5 µm intervals throughout the z axis, using a Nikon Fluor × 10 lens (numerical aperture = 0.5) with an electronic zoom of × 8 and a theoretical resolution of 0.6 µm. Each series of sequential z sections was processed to determine the maximum intensity value for each x,y coordinate (effectively collapsing the z dimension), and this information was used to calculate an extended focus image (PZ series) of the labelled areas throughout the blastocyst. The labelled areas were resolved into 256 grey levels of fluorescence intensity and the images presented in pseudocolour to emphasize the differences in fluorescence intensity. Smaller groups of sections for the embryo surface (sections 1–3) and midplane (sections 7–9) were also used.

Analysis of the diameters and number of endocytic vesicles in control and insulin-treated blastocysts was performed using the Interactive Biological Analysis System (IBAS; Kontron Elektronik Ltd, St Albans). Binary images of the stored confocal images (PZ series and smaller summed sections) were produced using the confocal software, to extract features from the background. These were captured on colour slide film and scanned into a video camera. An IBAS evaluation program was then used to analyse the number and diameters of vesicles in each image.

For each blastocyst, the IBAS provided a value for the total number of vesicles per blastocyst and a corresponding mean vesicle diameter per blastocyst ($\bar{x}_i$). A mean vesicle diameter $\pm$ SEM was then determined for 10–12 blastocysts per treatment as follows:

$$\text{mean} = \sum n_i x_i / \sum n_i$$

where n_i = number of vesicles per blastocyst and x_i = mean vesicle diameter per blastocyst.

$$\text{variance, } s^2 = [\sum n_i x_i^2 - (\sum n_i x_i)^2 / \sum n_i] / (\sum n_i - 1)$$

$$\text{SEM} = \sqrt{s^2 / \sum n_i}$$

As each PZ series was composed of approximately 12 sections, each 3 µm thick and about 5 µm apart through the

Fig. 1. (a) Confocal transmission image of a blastocyst. TE: trophoblast; ICM: inner cell mass; BC: blastocoel cavity; ZP: zona pellucida. (b–c) Pseudocoloured confocal images displaying equatorial sections ($\approx 3 \mu\text{m}$) of (b) blastocysts incubated in M16 containing $12.5 \text{ g FITC dextran l}^{-1}$ at 4°C , (c) blastocysts incubated in BMOC2 containing $12.5 \text{ g FITC albumin l}^{-1}$ at 37°C and (d) at 4°C for 2 h. Red represents high, and blue low fluorescence intensity. Scale bar represents $25 \mu\text{m}$.

embryo (i.e. a total thickness scanned of approximately $36 \mu\text{m}$) and the average diameter of blastocysts fixed to the chambers was $60 \pm 0.82 \mu\text{m}$, the number of vesicles counted per blastocyst should be multiplied by the factor $60/36$, to present the total number of labelled vesicles in the blastocysts accurately.

Statistical analysis

Statistical analysis of the differences between control and insulin-treated blastocysts was performed by Student's *t* test.

Electron microscopy

Freshly collected blastocysts (96 h after hCG injection) were incubated in BMOC2 in the presence or absence of $170 \text{ nmol insulin l}^{-1}$ at 37°C for 6 h, collected, washed extensively in M2 and placed onto fibroblast monolayers, which had been grown on Thermanox coverslips (Nunc Inc., IL) and exposed to $0.04 \text{ g phytohaemagglutinin (L9017; Sigma Chemical Company) l}^{-1}$ in M2 (Wiley *et al.*, 1985). The coverslips with attached embryos were flooded with 3% (w/v) glutaraldehyde in $0.066 \text{ mol cacodylate l}^{-1}$ buffer, pH 7.2 at 25°C , and left at this temperature for 30 min before fixation was completed at 4°C overnight. After washing with $0.066 \text{ mol cacodylate l}^{-1}$ buffer containing $0.15 \text{ mol sucrose l}^{-1}$, pH 7.2, the embryos were post-fixed with 1% (w/v) osmium tetroxide in $0.066 \text{ mol cacodylate l}^{-1}$ buffer, pH 7.2, before embedding in Spurr's

resin (Spurr, 1969). Sections were cut at 70 nm on a Nova ultramicrotome (LKB, Bromma), using a diamond knife (Diatome Ltd, Biene), and stained with 0.5% (w/v) uranyl acetate in 50% (v/v) ethanol and lead citrate (Reynolds, 1963), before examining in a Hitachi H800 electron microscope operated at 100 kV . Control and insulin-treated blastocysts were compared by viewing up to ten sections per blastocyst from 3–4 blastocysts per treatment over three experiments.

Results

Confocal microscopy

Blastocysts (see Fig. 1a for blastocyst morphology) incubated with FITC dextran for 2 h at 37°C revealed staining of endocytic vesicles in the trophectoderm, but not in the ICM or blastocoel cavity (Fig. 2f). No labelling of cells with FITC dextran was apparent at 4°C (Fig. 1b). At 37°C , the pattern of labelling with FITC albumin was similar to that with FITC dextran (Fig. 1c), although at 4°C FITC albumin was evident in the zona pellucida (Fig. 1d), a result never observed with FITC dextran.

In freshly collected blastocysts exposed to insulin for up to 6 h, there was a marked increase in the intensity of FITC dextran labelling of trophectoderm vesicles (Fig. 2a, f). Analysis of the effect of treatment with insulin for 2.5–6.0 h on mean vesicle diameter and number per embryo performed on PZ

Fig. 2. Pseudocoloured confocal images of (a, d) PZ series; (b, e) sections 1–3 (surface region); and (c, f) sections 7–9 (midplane region) images of blastocysts that had been incubated at 37°C in M16 (a–c) or M16 containing 170 nmol insulin l^{-1} (d–f) for 4 h, and then transferred to the same medium containing 12.5 g FITC dextran l^{-1} at 37°C for 2 h. Red represents high and blue low fluorescence intensity. Scale bar represents 25 μm .

series, surface region (sections 1–3) and midplane region (sections 7–9) images of control and insulin treated blastocysts (Fig. 3), showed that in control blastocysts, as the culture period increased from 2.5 h to 6 h, mean vesicle diameter per embryo decreased slightly, while mean vesicle number per embryo decreased more substantially (Fig. 3). Data from control blastocysts cultured from the late eight-cell stage were similar to those observed for blastocysts after control incubation for 6 h (Fig. 3).

Insulin treatment generally increased mean vesicle diameter per embryo in the PZ series and both surface and midplane section series, on average by 15% ($P < 0.05$; Fig. 3a). This was

evident in both freshly collected blastocysts and blastocysts cultured for 22 h from the late eight-cell stage. In contrast, after incubation with insulin for 2.5 h, the mean number of vesicles per embryo was reduced by 25% ($P < 0.01$) in the PZ series (Fig. 3b). However, by 4 h, more vesicles were present in insulin-treated than in control blastocysts in both PZ and section images, and this rose to 56% ($P < 0.01$) in the PZ and midplane series ($P < 0.01$) after exposure to insulin for 6 h. Culture of late eight-cell embryos to the blastocyst stage in insulin produced a 30% ($P < 0.01$) and 55% ($P < 0.001$) increase in mean number of vesicles per embryo in the PZ and surface series, respectively, compared with controls (Fig. 3b).

Fig. 3. Temporal effects of insulin (■) on (a) vesicle diameter and (b) number of vesicles per embryo in PZ series, sections 1–3 and 7–9 of confocal images of blastocysts incubated in M16 in the presence or absence of 170 nmol insulin l⁻¹ at 37°C for 2.5, 4 and 6 h. (□) Control values. Labelling with 12.5 g fluorescein isothiocyanate (FITC) dextran l⁻¹ occurred in the last 2 h of the incubation period. Late eight-cell embryos were cultured under the same conditions for 22 h, before reaching the blastocyst stage. Blastocysts, fresh and cultured, were then collected, washed and fixed for confocal microscopy and image analysis. Each data point represents the mean $\pm$ SEM, $n = 10$ –12 embryos, 5–6 embryos per treatment per experiment. Significantly different from control * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Electron microscopy

Compared with controls, blastocysts that had been pre-treated with insulin for 6 h showed increased membrane convolutions at the apical surface of trophectoderm cells,

manifested as ‘ruffling’ of the cell membrane and finger-like projections extruding from the membrane surface (Fig. 4). Some projections appeared to fold back or collapse towards the membrane, possibly owing to the restriction in movement imposed by the zona pellucida, suggesting the formation of

Fig. 4. Trophoblast (TE) membrane activity in (a) control and (b) insulin-treated blastocysts. Blastocysts were cultured in BMOC2 with and without $170 \text{ nmol insulin l}^{-1}$ at 37°C for 6 h, and then collected and prepared for electron microscopy using conventional methods. Arrowheads indicate the formation of finger-like projections that have folded towards the trophoblast membrane (TE), and the arrow indicates possible macropinosome formation. ZP: zona pellucida. Scale bar represents $1 \mu\text{m}$.

large vesicles or macropinosomes. Perhaps because of this ruffling, the zonae pellucidae of insulin-treated blastocysts were not as closely apposed to the trophoblast plasma membrane as those of control blastocysts.

Discussion

In this study, we have used confocal fluorescence microscopy combined with a morphometric image analysis programme and

electron microscopy, to characterize and quantify the effect of insulin on endocytosis in mouse blastocysts. One consistent observation, whether using the fluid phase marker, FITC dextran, or the combined membrane and fluid marker, FITC albumin, was that labelling occurred exclusively in the outer trophoblast cells of the blastocyst. Uptake of the two fluorescent markers was inhibited at low temperature, but at 37°C , both displayed a punctate distribution, typical of cytoplasmic vesicles, in the trophoblast. After incubation for 2.5 h, the labelled sites were $2.5\text{--}3.0 \mu\text{m}$ in diameter, which is

consistent with the size of multivesicular bodies and lysosomes in confocal images, but is possibly overestimating true diameter by about 1 μm , because of the optics of the confocal system. These size estimates are in agreement with those of earlier microscope studies of blastocysts (Chisholm *et al.*, 1985; Fleming and George, 1987) and support those from earlier studies on embryos of rats (Schlafke and Enders, 1973), rabbits (Hastings and Enders, 1974), mice (Fleming, 1986; Fleming and Goodall, 1986; Pemble and Kaye, 1986; Dungleison and Kaye, 1993, 1995) and sheep (Pullar *et al.*, 1990). Collectively, they demonstrate that the mammalian trophectoderm engages in active endocytosis of external fluid, not only for direct transcytosis, but also for lysosomal breakdown and the supply of low molecular weight nutrients to support other embryonic processes in both the trophectoderm and possibly ICM tissues, after release to the blastocoel. Confocal microscopy showed albumin, but not dextran, binding to the zona pellucida, particularly at 4°C; this is consistent with albumin being an adsorptive and fluid phase marker, and dextran being a fluid phase marker.

Insulin treatment significantly increased the overall intensity of FITC dextran-labelled vesicles in the trophectoderm, which could be explained either by (i) a higher concentration of the marker within vesicles of the same size and frequency, or (ii) more fluid present within larger vesicles or an increased number of vesicles per unit volume. In (i), a higher concentration could be achieved by increased membrane binding before endocytosis but, for FITC dextran, there is no evidence that surface binding takes place. These possibilities were analysed by quantifying the mean vesicle diameter and number of vesicles per blastocyst from PZ series and section images of insulin-treated and control blastocysts. The analysis showed that in freshly collected blastocysts exposed to insulin for 2.5–6 h, there was a significant increase of 15% in mean vesicle diameter ($P < 0.05$). This increase was not distorted by vesicle 'overlap' in the PZ series, as it was also apparent in the surface and midplane series (Sections 1–3 and 7–9 in Fig. 3). In addition, insulin caused a significant increase of 56% ($P < 0.01$) in number of vesicles per embryo in the PZ and midplane series images, but only after incubation for 6 h.

The increase in trophectoderm vesicle diameter and number after insulin treatment probably explains the overall increase observed in vesicle intensity. These data together with our analysis of insulin stimulation of uptake of ^{125}I -labelled BSA (Dungleison and Kaye, 1993) and ^3H -dextran (Dungleison and Kaye, 1995) lead to the conclusion that insulin acts on the endocytic apparatus in trophectoderm to stimulate the internalization of surrounding fluid.

The increase in vesicle diameter could represent a membrane phenomenon whereby the size of pinocytic vesicles pinching off from the apical surface is increased. This has been observed in macrophages after stimulation with phorbol ester (Swanson, 1989), and in human carcinoma A431 cells in response to epidermal growth factor (Haigler *et al.*, 1979). Alternatively, the increase in vesicle diameter could reflect a cytoplasmic event where vesicle fusion is enhanced, possibly leading to larger multivesicular bodies or lysosomes. This activity has also been observed in macrophages after growth factor stimulation (Swanson *et al.*, 1987; Nilsson *et al.*, 1989). In this case, the number of vesicles would decrease proportionally with vesicle

fusion. Consistent with this, analysis of our data showed that after insulin treatment for 2.5 h, mean vesicle diameter was significantly increased, while the number of vesicles was significantly lower than in controls. However, extensive morphometric analysis of endosomes, multivesicular bodies and lysosomes in electron microscopy sections is required to confirm this.

After longer incubations with insulin, mean vesicle diameter and number of vesicles were both increased compared with controls. Here, cytoplasmic vesicle fusion alone cannot explain the results, and increased membrane activity is implicated. In the ultrastructural analysis, insulin treatment appeared to increase trophectoderm apical membrane convolutions, and irregular fluctuations and finger-like projections were frequently apparent. In some cases, these projections appeared to fold back on themselves, resembling the formation of macropinosomes. This may be a direct effect of insulin to increase pinocytic activity at the surface, or simply due to the constriction by the zona pellucida. Furthermore, other pathways, including coated and uncoated pits and cavaeolae, may contribute to macropinosome formation after long-term treatment with insulin. Further ultrastructural morphometric studies are required to confirm this observation, but it lends support to our conclusion that the increased vesicle fusion alone cannot explain the increased size and number of vesicles present in blastocysts after exposure to insulin for longer. Membrane ruffling leading to macropinosome formation has been observed after growth factor stimulation of various cell types (Brunk *et al.*, 1976; Bar-sagi and Feramisco, 1986; Kadowaki *et al.*, 1986; Miyata *et al.*, 1988). Taken together, our confocal, ultrastructural and kinetic data (Dungleison and Kaye, 1993, 1995) suggest that, from 6 h onwards, insulin stimulates trophectoderm membrane activity and micropinosome formation, leading to an increased uptake of endocytosed fluid and membranes localized within larger and more numerous endocytic vesicles compared with controls. Similar stimulations in mean vesicle diameter and number per embryo were obtained for blastocysts that had been cultured from the late eight-cell stage. However, embryos exposed to insulin in long-term culture also show stimulated mitogenesis (Harvey and Kaye, 1990) and morphological development (Gardner and Kaye, 1991), so that the effects of insulin on endocytic activity in cultured embryos may also relate to its influence on other embryonic processes.

In the absence of insulin, blastocysts that were cultured for 4 h or longer showed a gradual, slight decline in the diameter and number of lysosomal bodies; this indicated that culture conditions may retard endocytic activity, which is consistent with their effects on other embryonic processes (Bowman and McLaren, 1970; Anderson and Foote, 1975; Turner *et al.*, 1992, 1994). These effects appear to be eliminated in the presence of insulin, supporting the conclusions of the beneficial effects of this growth factor on blastocyst development *in vitro* (Kaye *et al.*, 1992).

Mouse reproductive tract fluids contain 10 g protein l^{-1} (Gardner and Leese, 1990), which is likely to provide a major source of amino acids for protein synthetic processes after endocytic uptake and lysosomal hydrolysis. As this report shows, insulin, also present *in utero* (Heyner *et al.*, 1989), stimulates this process considerably, increasing the delivery of

protein and other macromolecular nutrients to support cell proliferation, differentiation and blastocyst expansion. The separate contributions of fluid phase and membrane bound components to the endocytosis of albumin have been assessed, using radiolabelled tracers, to be about equal at 10 g l^{-1} , delivering a total of about 3.4 ng h^{-1} (Dunglison and Kaye, 1995). If mouse uterine albumin resembles human serum albumin and contains 14 glycine residues per molecule, the fluid phase endocytosis visualized in this study would deliver about $300 \text{ fmol glycine h}^{-1}$ to the blastocyst as albumin. This is about 10% of that available through amino acid transport (Hobbs and Kaye, 1985), assuming uterine glycine concentrations are saturating and there is no competition by other uterine amino acids for uptake. Another factor is the potential of endocytosed albumin to carry such nutrients as Cu^{2+} , fatty acids, sterols and tryptophan. These data reveal the potential nutritional role of maternal proteins, embryonic endocytosis and lysosomal activity for the preimplantation embryo.

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