myo-Inositol transport and metabolism in fetal-bovine aortic endothelial cells

Gerard T. BERRY,* Roy A. JOHANSON, J. Eric PRANTNER, Beatrice STATES and John R. YANDRASITZ

Division of Biochemical Development and Molecular Diseases, Department of Pediatrics, University of Pennsylvania School of Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, U.S.A.

The *myo*-inositol transport system in confluent fetal-bovine aortic endothelial cells was characterized after 7–10 days in subculture, at which time the *myo*-inositol levels and rates of *myo*-[2-³H]-inositol uptake and incorporation into phospholipid had reached steady state. Kinetic analysis indicated that the uptake occurred by both a high-affinity transport system with an apparent K_t of 31 μ M and V_{max} of 45 pmol/min per mg of protein, and a non-saturable low-affinity system. Uptake was competitively inhibited by phloretin, with a K_i of 50 μ M; phloretin was a non-competitive inhibitor, with half-maximal inhibition between 0.2 and 0.5 mM. Glucose was a weak competitive inhibitor, with a K_i of 37 mM; galactose failed to inhibit uptake. A weak dependence on Na⁺ for the initial rate of uptake was observed at 11 μ M *myo*-inositol. When fetal-bovine serum (FBS)-supplemented medium, which contained 225 μ M *myo*-

inositol, was used, the cells contained about 200 nmol of *myo*-inositol/mg of DNA. With adult-bovine-serum (ABS)-supplemented medium, which contained 13 μ M *myo*-inositol, the cells contained about 110 nmol/mg of DNA. Transport of 11 μ M *myo*-[2-³H]inositol was 18 and 125 pmol/min per mg of DNA for cells grown in FBS and ABS respectively. Kinetic analysis showed that for the cells grown in FBS the V_{max} of the high-affinity system was decreased by 64%, whereas the K_t remained essentially unchanged. Increased cell *myo*-inositol levels were not associated with an increased rate of phosphatidylinositol synthesis. After prolonged exposure of fetal endothelial cells to a *myo*-inositol concentration which approximated to a high fetal as opposed to a low adult blood level, cell *myo*-inositol levels doubled and high-affinity transport underwent down-regulation.

INTRODUCTION

myo-Inositol levels in most mammalian cells or tissues are 5–500-fold higher than the low micromolar concentrations in plasma and interstitial fluids (Dawson and Freinkel, 1961; Lewin et al., 1976). Active transport systems with high affinities for *myo*-inositol are thought to be responsible for the maintenance of these concentration gradients (Cotlier, 1970; Spector and Lorenzo, 1975; Greene and Lattimer, 1982; Biden and Wollheim, 1986). For unknown reasons, *myo*-inositol concentrations in fetal mammalian serum may be elevated to levels which are sometimes greater than 1 mM, depending on the time of gestation (Campling and Nixon, 1954; Battaglia et al., 1961; Hallman et al., 1986). In general, however, the increase in fetal serum compared with adult serum is not paralleled by a comparable increase in fetal tissue *myo*-inositol levels (Battaglia et al., 1961; Hallman et al., 1986). Since tissue *myo*-inositol levels are usually greater than 1 mM when ambient concentrations are 10–70 μ M, and the K_m of CDP-1,2-diacyl-*sn*-glycerol:*myo*-inositol 3-phosphatidyltransferase (EC 2.7.8.11) for *myo*-inositol in fetal tissue is in the low-micromolar range (Bleasdale et al., 1979), one would not expect that a 10–100-fold increase in extracellular fetal levels would affect the rate of synthesis of PtdIns, the most abundant *myo*-inositol-containing phospholipid. Furthermore, as little as 4 μ M *myo*-inositol was reported to decrease phosphatidylglycerol synthesis by 50% in microsomes from fetal lung, presumably because of competition for CDP-diacylglycerol (Hallman et al., 1986). Using rat pancreatic islets, an analogous effect of micromolar concentrations of *myo*-inositol on PtdIns

synthesis was first observed by Freinkel et al. (1975). One possible explanation for the high levels of *myo*-inositol in the fetal circulation is that one or more *myo*-inositol transporters in certain fetal cells require millimolar concentrations of *myo*-inositol to maintain adequate transport. Theoretical mechanisms that might account for such atypical kinetics include the absence of a high-affinity transport component, dissociation of co-transport with Na⁺ or a decreased electrochemical gradient for Na⁺. Another possibility, but of a different nature, is that in the fetus, with a poorly developed circulation, *myo*-inositol levels in deep tissues are maintained by passive diffusion, thus requiring an elevated concentration of *myo*-inositol at the site of the vascular endothelium.

Cultured cells from various tissues and species have been used to study the kinetics of *myo*-inositol transport (Segal et al., 1984; Hallman et al., 1986; Li et al., 1986; Yorek et al., 1986, 1987; Khatami and Rockey, 1988; Nakanishi et al., 1989; Yorek and Dunlap, 1989; Haneda et al., 1990; Fruen and Lester, 1990; delMonte et al., 1991), yielding results comparable with those obtained in the intact tissue. Both adult bovine aortic and pulmonary endothelial cells have been shown in culture to display active transport systems for *myo*-inositol that are Na⁺-dependent and of the high affinity type (Yorek et al., 1986; Yorek and Dunlap, 1989). In this study, we utilized cultured fetal-bovine aortic endothelial cells (BAEC) to delineate the nature of *myo*-inositol transport in the fetal endothelial cell and to determine the effect of serum derived from either the fetal or the adult animal on steady-state *myo*-inositol levels, the rate of uptake of *myo*-inositol and the rate of synthesis of PtdIns.

Abbreviations used: BAEC, bovine aortic endothelial cells; FBS, fetal-bovine serum; ABS, adult bovine serum; CBS, calf bovine serum; MEM, Minimum Essential Medium with Earle's balanced salts.

* To whom correspondence should be addressed.

EXPERIMENTAL

Materials

BAEC were obtained from the Coriell Institute for Medical Research, Camden, NJ, U.S.A. Two fetal cell lines, AG-07680 and AG-07684, and one adult cell line, AG-8132, were utilized in the experiments. Minimum Essential Medium with Earle's balanced salts (MEM) was obtained from JRH Biosciences, Lenexa, KS, U.S.A. Fetal-bovine serum (FBS) was from Whittaker Bioproducts, Walkersville, MD, U.S.A., and calf bovine serum (CBS; cat. no. A-2151) was from Hyclone Laboratories, Logan, UT, U.S.A. Adult bovine serum (ABS) and other biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. Polystyrene tissue-culture flasks were made by Corning Glass Works, Corning, NY, U.S.A. Radioisotopes were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. G.l.c. reagents and column packings were from Supelco, Bellefonte, PA, U.S.A. Silica-gel H t.l.c. plates were from Analtech, Newark, DE, U.S.A.

Cell culture

The fetal BAEC were grown in 25 cm² culture flasks containing MEM and 20% FBS supplemented with 2 mM glutamine in a 37 °C incubator containing 5% CO₂ in humidified air. The stock MEM contained 11 µM *myo*-inositol. The cells were subcultured (1:10) weekly and refed at least twice per week. Lines AG-7680 and AG-7684 were studied between passages 8 and 17. For experimental studies, the cultured cells were fed with 9 ml of MEM, either unsupplemented or supplemented with 20% FBS, 20% ABS or 15% CBS on days 1, 5 and 8. When indicated, the cells were subcultured in MEM supplemented with 20% FBS, allowed to recover for 2 days, and then transferred to serum-free MEM with a refeeding on day 4.

Preparation and assay of cell extracts

On the days indicated in the Results section, cell extracts were prepared for determination of DNA, protein or *myo*-inositol content. The flasks were removed from the incubator, the media were gently aspirated, and cells were washed twice at room temperature with Dulbecco's PBS without Ca²⁺ and Mg²⁺. The cells were then lysed by adding 2 ml of ice-cold water and placing the flasks on ice for at least 1 min. The lysates were scraped and transferred to tubes along with a 2 ml water rinse of the flask. For measurement of *myo*-inositol by g.l.c. as the trimethylsilyl derivative, an internal standard of 20 nmol each of ribitol and α -methylmannopyranoside was added to each lysate. Samples of the suspension were taken for determination of protein and DNA. The remainder was centrifuged at 40 000 *g* for 15 min at 4 °C. The supernatant was recovered, and either a portion was used directly or the entire fraction was evaporated in a SpeedVac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.).

For determination of *myo*-inositol by g.l.c., the trimethylsilyl derivatives of dried samples were prepared by stirring overnight with 80–100 µl of pyridine/bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (20:20:1, by vol.) at room temperature. Chromatography was performed with a Perkin–Elmer model 3920 instrument with a flame ionization detector. Electronic integration of the detector signals was performed by a Spectra-Physics SP4000 integrator (Spectra-Physics, San Jose, CA, U.S.A.). Separations were accomplished in a 1.83 m (6 ft) × 4 mm glass column packed with 3% SP-2100 on Supelcoport.

Uptake and incorporation of *myo*-inositol

Experiments on the uptake of *myo*-[2-³H]inositol were performed on confluent cells in MEM without serum to which was added a sterile mixture of *myo*-[2-³H]inositol and [¹⁴C]poly(ethylene glycol)-4000 with a tracer ratio of about 5:1. For concentration-dependence experiments, various amounts of a sterile 50 mM *myo*-inositol stock solution were added; the amount of label was increased at the higher concentrations of *myo*-inositol to offset partially the decrease in specific radioactivity. Glucose and galactose were added as sterile 0.5 M solutions. Phlorrhizin and phloretin were added as 50 mM solutions in 50% ethanol. Ethanol alone at up to 1% concentration had no effect on the uptake of *myo*-[2-³H]inositol. For Na⁺-dependence studies, the media were prepared by mixing normal MEM with MEM in which the NaCl had been replaced by choline chloride (Gibco Laboratories, Grand Island, NY, U.S.A.).

To initiate the incubation, flasks were removed from the incubator. The medium was gently aspirated, 4–5 ml of fresh medium containing radioisotopes and additions were added, and the flasks were returned to the incubator for the indicated times. At the end of the incubation, the medium was removed and cells were washed twice at room temperature with PBS. Except where indicated, cells were lysed by adding 2 ml of ice-cold water, and the lysates prepared and centrifuged as described above. Membrane pellets were washed once by resuspending in 4 ml of cold water and re-centrifuged.

Samples were counted for radioactivity in Ecolite (ICN Biomedicals, Irvine, CA, U.S.A.) at 19 parts of Ecolite to 1 part of water in a Packard Tri-Carb 2000CA liquid-scintillation analyser. Triplicate 10 µl samples of the labelled incubation media were counted for radioactivity to obtain a ¹⁴C/³H ratio for calculation of trapped extracellular space and to calculate the specific radioactivity of the *myo*-[2-³H]inositol by using the known concentration of *myo*-inositol in the medium. The pmol of *myo*-inositol transported from extracellular fluid into the BAEC, either present in the soluble fraction or incorporated into membrane phospholipid, were calculated from the radioactivity content of both fractions, the specific radioactivity of *myo*-[2-³H]inositol in extracellular fluid and the correction factor for trapped extracellular fluid. In order to determine the distribution of *myo*-[2-³H]inositol in the membrane phospholipids, lipid extracts were prepared and analysed by t.l.c. Cells were incubated for 5–24 h in MEM with 11 µM *myo*-[2-³H]inositol, rinsed with PBS, and lysed with cold 10% trichloroacetic acid rather than cold water. The membrane pellets were subjected to lipid extraction and partition with acidified solvents, and phospholipids were separated by t.l.c. on potassium oxalate-impregnated silica-gel plates. PtdIns accounted for approx. 90% of the *myo*-[2-³H]inositol incorporated into membrane lipid, PtdIns4P 4–5%, and PtdIns(4,5)P₂ 5–6%. This lipid distribution was constant from 5 to 24 h of incubation.

Protein was measured by the method of Lowry et al. (1951). DNA was measured as described by Fiszer-Szafarz et al. (1981). Kinetic parameters were derived by non-linear-regression analyses of data by using SigmaStat (Jandel Scientific, Corte Madera, CA, U.S.A.) and Microsoft Excel 4.0 (Microsoft Corp., Redmond, WA, U.S.A.).

RESULTS

Characteristics of fetal BAEC growth

The effects of time and serum on the subcultured fetal BAEC DNA and protein content, two parameters of cell growth, are

Table 1 Effect of period of subculture and of serum on fetal BAEC growth, *myo*-inositol levels, uptake and incorporation into phospholipid

Fetal BAEC were split 1:10 into MEM containing 20% FBS or ABS and grown in 25 cm² flasks. After an overnight recovery, the medium was changed and the cells were grown for the indicated number of days, with changes of medium on days 4 and 7. The medium was removed, and cells were rinsed with PBS, lysed with cold water, and analysed for protein, DNA and *myo*-inositol levels, and uptake and incorporation into phospholipid of 11 μ M *myo*-[2-³H]inositol at 1 h. Several *myo*-inositol determinations performed on day 9 are included in day-10 results. The number of determinations is shown in parentheses. Except for DNA/protein data, all differences between corresponding FBS and ABS data are significant at $P < 0.01$ – 0.00001 . * $P < 0.05$ with respect to 5 versus 7 days in FBS; † $P < 0.01$ with respect to 5 versus 10 days in FBS.

Parameter	Serum	Day 5	Day 7	Day 10
DNA (μ g/flask)	FBS	158 $\pm$ 17 (6)	229 $\pm$ 36 (6)	209 $\pm$ 24 (5)
	ABS	57 $\pm$ 7 (8)	57 $\pm$ 8 (8)	54 $\pm$ 4 (8)
Protein (mg/flask)	FBS	3.17 $\pm$ 0.32 (6)*†	5.66 $\pm$ 0.95 (6)	7.16 $\pm$ 1.21 (5)
	ABS	1.41 $\pm$ 0.09 (8)	1.30 $\pm$ 0.11 (8)	1.22 $\pm$ 0.05 (8)
DNA/protein	FBS	55 $\pm$ 11 (6)	49 $\pm$ 12 (6)	32 $\pm$ 5 (5)
	ABS	41 $\pm$ 4 (8)	43 $\pm$ 3 (8)	45 $\pm$ 4 (8)
<i>myo</i> -Inositol (nmol/flask)	FBS	25 $\pm$ 3 (6)	32 $\pm$ 3 (10)	35 $\pm$ 7 (6)
	ABS	5 $\pm$ 2 (4)	4 $\pm$ 0.3 (4)	5 $\pm$ 0.6 (4)
Uptake (pmol/min per mg of DNA)	FBS	19.65 $\pm$ 0.96 (4)*	16.48 $\pm$ 0.42 (4)	16.55 $\pm$ 1.46 (4)
	ABS	124.4 $\pm$ 12.40 (4)	132.62 $\pm$ 19.02 (4)	117.38 $\pm$ 3.46 (4)
Incorporation (pmol/min per mg of DNA)	FBS	1.42 $\pm$ 0.09 (4)	1.32 $\pm$ 0.04 (4)	1.51 $\pm$ 0.33 (4)
	ABS	5.98 $\pm$ 1.69 (4)	6.14 $\pm$ 1.89 (4)	8.54 $\pm$ 0.65 (4)

presented in Table 1. The cells were split 1:10 into medium containing 20% FBS and allowed to recover overnight. They were then cultured for 10 days with media containing either 20% FBS or 20% ABS, with refeedings on days 4 and 7. Cultures grown in ABS-supplemented media had achieved their final DNA and protein contents by day 5 of the study, whereas the cells exposed to FBS-supplemented media continued to grow until day 7; even though the DNA levels on days 5 and 7 or 10 were not significantly different, the protein content was lower on day 5 compared with day 7 or 10. Thus the cells under either condition had reached confluence by at least day 7 in subculture and remained in a quiescent state until at least day 10. The growth of cells was approx. 3.5 times greater in the FBS-supplemented medium, as reflected in the amount of DNA per flask. On day 10, the contents of DNA and protein in the FBS-supplemented cells were 3.9- and 5.9-fold higher, respectively, than in the ABS-supplemented cells. The ratio of DNA to protein was independent of time and serum type, and overall was 45 μ g of DNA/mg of protein with FBS and 43 μ g of DNA/mg of protein with ABS exposure. There was a trend, however, in the cells cultured in FBS-supplemented media only for the DNA/protein ratio to decrease as protein content increased. As discussed below, the content of *myo*-inositol per flask also appeared to parallel the protein content. Not shown in Table 1 are other studies in which cells were split into FBS-supplemented medium, allowed to recover for 2 days, and then grown for another 7 days in MEM without serum with a change of medium at day 4. Cells grown in MEM without serum for 7 days after harvesting and re-attachment had the same DNA and protein contents on days 5 and 7, with an overall content of 1.09 $\pm$ 0.12 mg of protein/flask ($n = 8$) and 61.4 $\pm$ 5.6 μ g of DNA/flask ($n = 8$), similar to cultures grown in ABS-supplemented media. In one series of experiments, the adult BAEC line AG-8132 was studied. Comparable with the fetal cells, they too achieved confluence by at least day 7 in either FBS- or ABS-supplemented media. The adult cells also manifested increased growth with increased DNA and protein levels with FBS as opposed to ABS exposure.

Effect of confluence and serum on *myo*-inositol levels

Parameters of *myo*-inositol metabolism differed markedly,

depending on the type of serum present in the growth medium. Most batches of FBS contain about 1 mM *myo*-inositol, whereas ABS and CBS contain less than 50 μ M *myo*-inositol. The *myo*-inositol concentration of the FBS-supplemented medium was 225 $\pm$ 6 μ M ($n = 36$), whereas that of the ABS-supplemented medium was 13.2 $\pm$ 0.8 μ M ($n = 39$). The amount of *myo*-inositol per flask was approx. 6.5 times higher for BAEC grown in FBS-supplemented medium, whereas the level of protein per flask was about 4-fold higher. Overall, *myo*-inositol content seemed to follow protein content more closely. There was, however, no statistically significant difference in cellular *myo*-inositol content on days 5–10 under either condition, with overall levels of 0.11 $\pm$ 0.02 ($n = 12$) and 0.20 $\pm$ 0.02 ($n = 22$) nmol of *myo*-inositol/ μ g of DNA or 3.70 $\pm$ 0.47 ($n = 12$) and 11.21 $\pm$ 1.31 ($n = 22$) nmol/mg of protein in ABS- and FBS-supplemented cells respectively. Not shown in Table 1 is the *myo*-inositol content of the serum-free cultures which were analysed on days 5 and 7. Despite the *myo*-inositol concentration in the basic medium of only 11 μ M, there were 13 $\pm$ 4 ($n = 4$) and 16 $\pm$ 4 ($n = 4$) nmol of *myo*-inositol/flask or 0.19 $\pm$ 0.05 and 0.23 $\pm$ 0.04 nmol of *myo*-inositol/ μ g of DNA on day 5 and 7 respectively. Even though the duration of exposure to FBS was brief and the growth characteristics were identical with the effects with ABS exposure, these cellular levels are comparable with those associated with FBS exposure.

In one other experiment, cells were split into MEM containing 20% CBS to which was added supplemental *myo*-inositol, and grown for 10 days with changes of medium on days 4 and 7. The *myo*-inositol content of cells grown in the control medium (17 μ M *myo*-inositol) was 5.4 $\pm$ 0.1 nmol/mg of protein (mean $\pm$ range of 2 determinations). In medium containing 105 μ M *myo*-inositol, the cellular *myo*-inositol content was 6.7 $\pm$ 0.3, and in medium containing 209 μ M *myo*-inositol the level was 8.2 $\pm$ 0.8 nmol/mg of protein. That is, cells grown in CBS-supplemented media maintained a *myo*-inositol level similar to that found in cells grown in ABS. Cells grown in a medium with a *myo*-inositol content similar to that found in FBS-supplemented MEM contained about 50% more *myo*-inositol at the end of 10 days, a level close, but not equal, to the result obtained with FBS-supplemented media. There was no effect of passage number on the level of *myo*-inositol in BAEC.

Figure 1 Time course of *myo*-[2-³H]inositol uptake and incorporation by fetal BAEC

The day before the experiment, the medium was changed to MEM without serum. Incubations were started by replacing the medium with MEM containing 11 μ M *myo*-[2-³H]inositol and returning the culture flasks to the incubator for 2–24 h. After removal of the radioactive medium, and rinsing with PBS, the cells were lysed with cold water and the extract was centrifuged at 40 000 *g* for 15 min. The pellet was washed by resuspending in water and re-centrifuging. The supernatant and the washed pellet were counted for radioactivity, and *myo*-inositol uptake was calculated in pmol by using the specific radioactivity of the medium. Correction was made for extracellular radioactivity with ¹⁴C-PEG-4000. Results are means $\pm$ S.E.M. for 6–10 determinations.

Uptake and incorporation of *myo*-inositol into soluble and lipid fractions

The uptake of *myo*-[2-³H]inositol from media containing 11 μ M *myo*-inositol was examined in confluent fetal BAEC (see Figure 1). Uptake into the soluble fraction was approximately linear for the first 6 h, but was slower at later times. Fitting the data to a first-order equation indicated a limit of 6 ± 2 nmol/mg of protein. There was essentially no incorporation of label into membranes for the first 1 h, but then incorporation progressed linearly for 24 h. After 24 h of incubation, the total cellular radioactivity was about equally divided between the soluble and membrane fractions.

Kinetics of *myo*-inositol transport

The concentration-dependence of uptake was examined in 60 min incubations of confluent cells with *myo*-[2-³H]inositol at concentrations of 11–2011 μ M. The effect of growth in either ABS- or FBS-supplemented media on the kinetic analysis was also determined. Just before initiation of the uptake experiment using serum-free MEM, the cells were in either ABS- or FBS-supplemented media. As shown in Figure 2, the uptake of *myo*-inositol, particularly at low micromolar concentrations, was substantially decreased in BAEC cultured in FBS compared with ABS. For both conditions, the data suggest the presence of at least two *myo*-inositol transport systems. Non-linear least-squares regression to a two-system model with a high-affinity and a low-affinity transporter yielded an apparent K_t and V_{max} in molar and nmol/min per mg of protein units, respectively, for the low-affinity system. For practical purposes, therefore, there was no demonstration of a saturable second component. The better fit was to a high-affinity system with a diffusion component. For the cells grown in ABS, the apparent K_t was 31 μ M, the V_{max} 45 pmol/min per mg of protein and the apparent K_d 0.024 l/min per mg of protein. For the cells grown in FBS, the

Figure 2 Concentration-dependence of *myo*-[2-³H]inositol uptake by fetal BAEC

Endothelial cells were incubated with various concentrations of *myo*-[2-³H]inositol in MEM without serum for 60 min. Up to the time of the experiment, the cells were grown to confluence in ABS- or FBS-supplemented media and maintained in their respective media. (a) $\bullet$, Data for BAEC exposed to ABS; $\circ$, FBS exposure. Results are means $\pm$ S.E.M. for four determinations from two experiments performed in duplicate. The data were fitted by non-linear least-squares regression analysis (SigmaStat, Jandel Scientific) to a two-system model with a saturable and a non-saturable diffusion component. (b) The data shown as an Eadie-Hofstee plot; also shown are the lines that describe the calculated data, but for only the saturable component, the high-affinity system. The continuous line represents the kinetics for BAEC in ABS and the broken line that in FBS.

apparent K_t and K_d appeared unchanged at 26 μ M and 0.018, respectively, whereas the V_{max} was decreased by 64%, to 16 pmol/min per mg of protein.

The Na^+ -dependence of *myo*-inositol transport by BAEC was examined by using incubation media consisting of mixtures of normal MEM and MEM in which NaCl had been replaced by choline chloride. In the first series of experiments, the uptake of several concentrations of *myo*-[2-³H]inositol was measured for a 30 min period. Rates were normalized to the average values of the samples in 134 mM Na^+ and 11 μ M *myo*-inositol for each experiment. As seen in Figure 3, decreasing the Na^+ concentration in the medium by 50% had little effect on *myo*-inositol uptake. The effects of a further decrease varied with the concentration of *myo*-inositol studied. At concentrations of *myo*-inositol above its K_t for transport, uptake was modestly increased in the lower- Na^+ media. A positive dependence of transport on Na^+ was seen only at the lowest *myo*-inositol concentration tested.

In the second series of experiments, the uptake of 11 μ M or 110 μ M *myo*-[2-³H]inositol was examined at different times of incubation in normal or MEM with a low Na^+ concentration. As seen in Figure 4, the uptake at 20 min was similar to that shown in Figure 3. However, the transport of *myo*-inositol ceased at about 1 h in the low- Na^+ medium. That is, there appeared to be a requirement for Na^+ in the medium to allow for the continued uptake of *myo*-inositol into fetal BAEC.

Figure 3 Na^+ -dependence of the initial rate of *myo*-inositol uptake

The uptake of *myo*-[2- ^3H]inositol by BAEC was measured for a 30 min period in media of different Na^+ concentrations. Rates were normalized to the average values of the samples in 134 mM Na^+ and 11 μM *myo*-inositol for each experiment. Points are means $\pm$ S.E.M. for six determinations. The lines are a linear-regression fit for 11 and 61 μM *myo*-inositol and for an exponential decay for 127 and 227 μM *myo*-inositol.

Figure 4 Time course of the uptake of *myo*-[2- ^3H]inositol at 23 and 134 mM Na^+

Endothelial cells were incubated with (a) 11 μM or (b) 110 μM *myo*-[2- ^3H]inositol in normal MEM which contained 134 mM NaCl, or a mixture of normal MEM and MEM in which NaCl was replaced by choline chloride to give a final Na^+ concentration of 23 mM. Uptake of *myo*-inositol was determined at various times from 30 to 240 min. The data presented are means $\pm$ S.E.M. for four determinations (two at 240 min). The lines were calculated by non-linear-regression analysis, assuming a first-order dependence with time.

The effects of the transport inhibitors phlorrhizin and phloretin were examined on the uptake of *myo*-[2- ^3H]inositol at 60 min. Phloretin appeared to be a non-competitive inhibitor; the uptake of both 11 μM and 110 μM *myo*-inositol was decreased by 50% at phloretin concentrations between 0.2 and 0.5 mM. Phlorrhizin was a competitive inhibitor of 11 μM or 110 μM *myo*-[2- ^3H]inositol uptake (Figure 5). A non-linear-regression analysis of the phlorrhizin data using a Michaelis-Menten equation for a single competitive inhibitor yielded a K_i of $50 \pm 18 \mu\text{M}$ for phlorrhizin.

Inhibition of *myo*-inositol uptake by glucose and galactose was examined. Galactose up to 20 mM did not inhibit the uptake of 11 μM *myo*-[2- ^3H]inositol. Glucose was a very weak com-

Figure 5 Inhibition of *myo*-inositol uptake by phlorrhizin

Uptake of 11 or 110 μM *myo*-[2- ^3H]inositol by endothelial cells was measured at 60 min in the presence of phlorrhizin at the indicated concentrations. Points are means $\pm$ S.E.M. for two to four determinations; the lines are from a linear-regression analysis of the data after a double-reciprocal transformation. Non-linear-regression analysis of all the data gave a K_i for phlorrhizin of 50 μM .

Figure 6 Inhibition of *myo*-inositol uptake by glucose

The uptake of 11–200 μM *myo*-[2- ^3H]inositol by endothelial cells was measured at 30 min in the presence of 5–20 mM glucose. Points are the mean $\pm$ range of four determinations at each condition. The lines are from a linear-regression analysis of the data after a double-reciprocal transformation. A non-linear-regression analysis of all the data gave a K_i of 37 mM for glucose.

petitive inhibitor, as shown in Figure 6. Raising the glucose concentration from 5 to 20 mM resulted in only about a 30% decrease in the uptake of 11 μM *myo*-[2- ^3H]inositol at 30 min. A non-linear-regression analysis of this data yielded a K_i of $37 \pm 16 \text{ mM}$ for glucose inhibition of *myo*-inositol transport.

Effect of confluence and serum on *myo*-[2- ^3H]inositol uptake and incorporation into PtdIns

In contrast with the approx. 2-fold difference in *myo*-inositol levels per cell DNA content, the rate of uptake of 11 μM *myo*-[2-

^3H inositol was about 7 times higher in the cells grown in ABS (Table 1). There was no change in the rate of uptake from 5 to 10 days in culture in the cells grown with ABS. However, in the cells grown in FBS-supplemented medium, the rate of *myo*-inositol uptake was 19% higher on day 5 compared with day 7. This may be related to the fact that these cells were still involved in active growth on day 5, as reflected in the increment in protein content from days 5 to 7. Overall, the transport of $11\ \mu\text{M}$ *myo*-[2- ^3H]inositol was approx. 18 pmol/min per mg of DNA for cells grown in FBS and 125 pmol/min per mg of DNA for cells grown in ABS.

As shown in Table 1, the apparent rate of incorporation of *myo*-[2- ^3H]inositol into PtdIns was almost 5-fold higher in the BAEC grown in ABS as compared with FBS. This rate, however, cannot be accurately determined without a direct measurement of the intracellular specific radioactivity of *myo*-inositol, which in turn depends on the level of *myo*-inositol and the uptake of label. The latter parallels, but does not exactly match, the relatively increased incorporation rate in ABS-exposed cells. Relative to DNA content, however, the *myo*-inositol content is decreased by half in these cells. It is possible that the actual rate of synthesis of PtdIns is equal in BAEC grown in either serum. It is also very likely that this rate is not increased in BAEC in FBS compared with ABS. There were no significant differences in incorporation rates on days 5–10 in BAEC with either exposure. In a manner analogous to the effects of FBS compared with ABS on fetal cells, the confluent adult cells also contained approximately twice as much *myo*-inositol after growth in FBS-supplemented media. Whereas the rates of uptake of $11\ \mu\text{M}$ *myo*-inositol and rate of incorporation into phospholipid were 23 ± 2 ($n = 6$) and 0.39 ± 0.04 ($n = 6$) pmol/min per mg of DNA respectively with FBS exposure, the corresponding rates were 72 ± 5 ($n = 6$) and 2.10 ± 0.46 ($n = 6$) pmol/min per mg of DNA respectively after ABS exposure. Similarly to the fetal cells, increased cellular levels of *myo*-inositol in adult BAEC were not associated with an increased rate of PtdIns biosynthesis. Prolonged exposure to the relatively elevated levels of *myo*-inositol in FBS was also associated with a relative decrease in adult BAEC uptake of *myo*-inositol at the low concentration of $11\ \mu\text{M}$. In two experiments on day-10 cells, we studied the effect of distribution of *myo*-[2- ^3H]inositol in phosphoinositides after a 6 h incubation with $11\ \mu\text{M}$ *myo*-inositol in MEM after growth of adult BAEC in either FBS- or ABS-supplemented media. T.l.c. was for analysis of label in phospholipids, revealing that PtdIns, PtdIns4P and PtdIns(4,5) P_2 contained 89–92, 4–5 and 4–6% of the label, respectively, whether the BAEC were grown in medium with FBS or ABS. Thus the type of serum appeared to have no qualitative effect on the distribution of label in the phosphoinositides.

DISCUSSION

Because of their propensity for achieving a quiescent growth state, the fetal BAEC in culture have proved to be quite suitable for metabolic studies, providing a window of observation of several days duration. The delineation of *myo*-inositol transport kinetics in fetal BAEC was performed at confluence, at which time the levels of *myo*-inositol as well as the rate of *myo*-inositol uptake have reached a steady state.

The uptake of *myo*-inositol by the BAEC was at a rate sufficiently slow such that neither *myo*-inositol nor inositol phospholipid was labelled to a constant specific radioactivity by 24 h. At that time the label appeared equally distributed between *myo*-inositol and PtdIns. The kinetic data showed that there are at least two *myo*-inositol transport systems in fetal BAEC, one

which may be simple diffusion and the other a high-affinity system with an apparent K_t of $31\ \mu\text{M}$ and a V_{max} of 45 pmol/min per mg of protein for cells grown in ABS. The values are comparable with those for other *myo*-inositol transport systems in several different types of tissue (Cotlier, 1970; Spector and Lorenzo, 1975; Greene and Lattimer, 1982; Biden and Wollheim, 1986; Hallman et al., 1986; Ohga et al., 1990) or cells (Segal et al., 1984; Yorek et al., 1986, 1987; Khatami and Rockey, 1988; Nakanishi et al., 1989; Yorek and Dunlap, 1989; Fruen and Lester, 1990; Haneda et al., 1990; delMonte et al., 1991) from diverse species. In particular, the kinetic parameters of high-affinity *myo*-inositol transport in our fetal BAEC grown in FBS are comparable with those generated in a study of adult BAEC, which when almost confluent after growth in media containing over $100\ \mu\text{M}$ *myo*-inositol displayed an apparent K_t for *myo*-inositol of $23\ \mu\text{M}$ and a V_{max} of 12 pmol/min per mg of protein (Yorek and Dunlap, 1989). As has been found with other *myo*-inositol transporters, we detected uptake to be competitively inhibited by phloretin with a K_i of $50\ \mu\text{M}$, whereas phloretin was a non-competitive inhibitor, with half-maximal inhibition observed between 0.2 and 0.5 mM. Glucose was only a weak competitive inhibitor, with a K_i of 37 mM. Galactose, as high as 20 mM, failed to inhibit *myo*-inositol uptake.

The dependence of *myo*-inositol transport on Na^+ was complex. Only the initial rate of *myo*-inositol uptake, at a low micromolar concentration, was dependent on the presence of Na^+ in the medium. In contrast, with high concentrations of *myo*-inositol, the initial rate of uptake was actually increased in media with Na^+ concentrations less than 61 mM. Furthermore, although the dependence of *myo*-inositol uptake on Na^+ was not a saturable process, *myo*-inositol uptake in only 23 mM Na^+ ceased after about 1 h. The nature of the dependency of *myo*-inositol uptake on Na^+ remains obscure. In studies of human skin fibroblasts obtained from subjects of different ages, we observed a similar dependence of *myo*-inositol uptake on Na^+ , but only at low *myo*-inositol concentrations.

The level of *myo*-inositol in cultured BAEC was dependent on the type of serum used in the culture media to promote cell growth. Endothelial *myo*-inositol levels in cells subcultured in media with FBS were approx. 2-fold higher than those exposed to adult serum. This appears to be simply related to the differences in *myo*-inositol concentrations in fetal and in adult serum, as the MEM with 20% FBS contained about 17 times more *myo*-inositol than did the MEM with 20% ABS. Selective addition of *myo*-inositol to media containing calf serum also resulted in dose-related increments in BAEC *myo*-inositol levels.

Our data suggest that prolonged exposure of fetal BAEC to a particular extracellular concentration of *myo*-inositol affects the steady-state rate of uptake of $11\ \mu\text{M}$ *myo*-inositol. When the cells were grown in ABS-supplemented media which contained *myo*-inositol in amounts typically found in adult bovine blood, the rate of *myo*-inositol uptake in unsupplemented MEM was about 7 times higher than in cells exposed to FBS. Since the concentration of *myo*-inositol in MEM with 20% FCS was only $225\ \mu\text{M}$, the use of a medium with *myo*-inositol levels which more closely simulate those in fetal blood from early gestation probably would have had even more dramatic effects on expression of the high-affinity *myo*-inositol transporter, which is the predominant system being tested when extracellular *myo*-inositol concentration is $11\ \mu\text{M}$. When the corresponding alterations in *myo*-inositol levels and uptake, and thus the corresponding intracellular specific radioactivity of *myo*-inositol, are taken into account, the rate of PtdIns biosynthesis, as reflected in the incorporation of labelled *myo*-inositol into phospholipid, was not increased in the cells grown in FBS

compared with ABS, despite the increase in cellular *myo*-inositol levels. It is unclear whether an increase in the intracellular *myo*-inositol concentration might increase synthesis of PtdIns *de novo*, since the concentration of *myo*-inositol is in the millimolar range and CDP-diacylglycerol is only present in minute amounts and could be rate-limiting. However, the true K_m for *myo*-inositol of CDP-diacyl-*sn*-glycerol:*myo*-inositol 3-phosphatidyltransferase, an enzyme which normally functions in a membrane bilayer, is still unknown. Earlier studies had actually shown that the K_m was in the 1.5–2.5 mM range when assays were performed in the presence of albumin or detergent (Benjamins and Agranoff, 1969; Takenawa and Egawa, 1977).

In summary, prolonged exposure of fetal BAEC to a *myo*-inositol concentration which more closely approximates to the fetal as opposed to the adult blood level is associated with a 2-fold increase in cell *myo*-inositol content and an apparent down-regulation of high-affinity transport, unaccompanied by a change in the affinity of the transporter for *myo*-inositol. Whether this decrease in uptake is mediated by functional alterations or by a simple decrease in number of high-affinity transporters remains to be determined. As regards the nature of fetal cells, our results show that an 'adult-type' high-affinity transporter is expressed in fetal endothelial cells and has no features which would clearly distinguish it from other reported high-affinity transporters. The diffusion component, particularly if it represents facilitated diffusion, may be important during fetal development, since the *myo*-inositol levels in the fetal bovine circulation are close to 1 mM. Up-regulation of a potentially energy-independent transporter function has been reported in one fetal system (Hallman et al., 1986). Perhaps not surprising, but important nonetheless to our understanding of *myo*-inositol metabolism, is the observation that the increase in cellular *myo*-inositol levels mediated by exposure to fetal serum was not accompanied by an increase in the rate of synthesis of PtdIns. The applicability of these findings to the intact fetal organism is of course unknown. It is important to note that cells in culture, particularly those which are not primary cultures, may begin to express biochemical or physiological effects which have no relevance to their function *in vivo*. With these shortcomings in mind, we may state that it is possible that certain fetal cells *in vivo*, such as these macrovascular endothelial cell types, contain, relative to the adult organism, persistently elevated levels of *myo*-inositol when bathed in fetal blood. Some or the majority of fetal tissues, however, may not contain relatively elevated levels of *myo*-inositol even though the blood concentration is increased (Battaglia et al., 1961; Hallman et al., 1986). This phenomenon remains unexplained. The additional application of a controversial hypothesis that in some fetal tissue only low micromolar concentrations of *myo*-inositol are sufficient to drive PtdIns biosynthesis (Bleasdale et al., 1979, 1981; Hallman et al., 1986) only serves to enhance the confusion surrounding fetal *myo*-inositol metabolism. Since (1) low levels of ambient *myo*-inositol may be adequate to maintain normal cellular PtdIns production and (2) cellular *myo*-inositol content and PtdIns synthesis are dissociated or at least not tightly linked, it is possible that *myo*-inositol is playing another role in certain fetal cells that is distinct from one of a simple substrate for phospholipid biosynthesis. Given our new understanding of the importance of *myo*-inositol in the osmoregulatory systems of some specialized renal and

central-nervous-system cells in the mature mammal (Thurston et al., 1989; Nakanishi et al., 1989; Kwon et al., 1992), an obvious candidate role is that of a cellular osmolyte in the fetal endothelium. From reported fetal BAEC volume/protein relationships (Rosen et al., 1981), we estimate that the fetal BAEC *myo*-inositol level is 1–2 mM and 4–6 mM when ECF contained 11 μ M and 225 μ M *myo*-inositol respectively. It is possible that, when ambient levels rise above 1 mM, the fetal BAEC levels will surpass 10 mM, a concentration which might confer some osmolar buffering capacity. Alternatively, the measured cellular level may not be equivalent to the free concentration or activity of *myo*-inositol in the BAEC, because a large fraction is bound to macromolecules or sequestered within organelles (Molitoris et al., 1980; Sigal et al., 1993), subserving roles in cell function that remain to be determined.

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