Protein degradation in cultured fetal hepatocytes

Absence of an inhibitory effect of insulin

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The role of insulin to regulate protein turnover in fetal liver was investigated using primary cultures of fetal-rat hepatocytes. The basal rate of protein degradation (in the presence of insulin and amino acids) was the same in cultured fetal and adult hepatocytes (2.48 ± 0.16 versus 2.46 ± 0.06 % of total protein degraded/h respectively). Incubation of cells in an unsupplemented media (without insulin or amino acids) resulted in a deprivation-induced increase in degradation in cells from both groups ($P < 0.05$). Rates of proteolysis could be returned to their respective basal values by the addition of amino acids at 5 times their normal plasma concentrations. In adult cells, addition of insulin alone significantly inhibited protein degradation ($P < 0.05$), whereas, in contrast, insulin was without effect on protein degradation in fetal hepatocytes. Both fetal and adults cells responded to dibutyl cyclic AMP with an increase in protein degradation above that seen in the no-additions group. Results of experiments in which the effect of inhibitors of protein degradation (chloroquine, NH_4Cl , amino acids and dinitrophenol) were tested suggested that lysosomes were responsible for 20–30 % of total protein degradation in fetal hepatocytes. Impaired insulin processing in fetal hepatocytes was examined as a possible cause of the insulin-resistance in these cells. As determined by h.p.l.c. analysis, the same pattern of initial degradation products of insulin was found in fetal hepatocytes as had previously been found in adult hepatocytes. Incubation of cells with various doses of chloroquine resulted in an increase in cell-associated ^{125}I -insulin and a decrease in insulin degradation in both fetal and adult cells. At the highest dose of chloroquine tested (500 μM), a slightly greater increase in insulin binding and a decrease in insulin degradation were observed in fetal cells as compared with adult cells. Rates of insulin internalization were also compared between fetal and adult cells. A 30 % slower rate of insulin internalization was observed in fetal cells, as compared with adult cells. It was concluded that the absence of an effect of insulin on protein degradation in fetal hepatocytes is not the result of a major difference in insulin internalization and processing between fetal and adult hepatocytes.

INTRODUCTION

Macrosomia in the developing fetus is a well-known consequence of poor diabetes control during pregnancy in women [1]. Fetal macrosomia is generally attributed to hyperinsulinaemia resulting from fetal hyperglycaemia, which in turn is due to hyperglycaemia in the maternal compartment [1]. A role for insulin in promoting fetal growth is suggested by studies in which insulin treatment *in utero* resulted in significant increases in body weights at birth [2,3].

Insulin is an important regulator of hepatic protein metabolism in the adult, but its exact role in regulating liver protein metabolism and liver growth in the developing fetus has not been established. In the rat, hepatic insulin receptors are present as early as day 14 of gestation [4] and are present in normal or higher than normal numbers at or near term [5–8]. However, the receptor/effector system for insulin in the fetal liver exhibits at least two differences as compared with the normal adult liver. First, fetal insulin receptors are not down-regulated in number, despite high circulating plasma insulin concentrations *in vivo* [4,9], or in experiments in which insulin is added in high concentrations to cultured cells *in vitro* [9,10]. Secondly, the fetal liver exhibits a partial resistance to insulin action [3,11–13].

The fetal liver at term consists primarily of parenchymal cells, but earlier in development the liver also contains significant numbers of haematopoietic cells [14,15]. The presence of haematopoietic cells in the liver can complicate the interpretation

of certain types of experiments, since these cells also contain insulin receptors [7]. We have established in our laboratory a method for the purification and culture of fetal hepatocytes [12] and, using this system, the effect of insulin and other agents on fetal hepatic protein degradation has been evaluated in the present studies. Although total protein degradation was responsive to a number of other agents, insulin was without effect in fetal hepatocytes. The absence of an effect of insulin on protein degradation in fetal cells did not appear to be the result of an overt abnormality in insulin processing in fetal cells, although they did display a slightly slower rate of insulin internalization as compared with cultured adult hepatocytes.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (150–200 g) or timed-pregnant (19–20 days) female Sprague–Dawley rats were obtained from Harlan Industries, Indianapolis, IN, U.S.A. All rats were given free access to food and water at all times.

Materials

Fetal-bovine serum, Leibovitz L-15 tissue-culture medium [16] and Earle's balanced salt solution [17] were from Grand Island Biological Co., Grand Island, NY, U.S.A. Bovine dermal collagen was from Flow Laboratories, McLean, VA, U.S.A.

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Collagenase (type II) was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and epidermal growth factor was from Collaborative Research, Bedford, MA, U.S.A. L-[2,3-³H]Valine was purchased from Amersham Corp., Arlington Heights, IL, U.S.A. ¹²⁵I(A14)-insulin was generously given by Dr. Bruce H. Frank, Eli Lilly and Co., Indianapolis, IN, U.S.A. Crystalline pig insulin (lot no. 615-2H2-3OD), pig glucagon, human growth hormone and human insulin-like growth factor-II were given by Dr. Ronald E. Chance, Eli Lilly and Co. All other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Fetal hepatocyte isolation and culture

Fetal-rat hepatocytes were isolated by the method described previously [12]. Hepatocytes were isolated under sterile conditions by collagenase digestion and were harvested by repeated centrifugation and washing. The final cell pellet was resuspended in 30–50 ml of Leibovitz L-15 culture medium, supplemented with proline and lysine to final concentrations of 0.93 mM and 2.2 mM respectively (hereafter referred to as fortified L-15). The initial viability was assessed by Trypan Blue exclusion and was generally 80–90% before plating. Batches of (2–3) × 10⁶ cells were plated in collagen-coated 25 cm² culture flasks in 5 ml of fortified L-15 medium containing 10 μM-cortisol and were incubated at 37 °C for 2 h. The attached cells were then washed with 2 × 2 ml of fresh fortified L-15 medium, followed by an overnight (16 h) incubation in fresh fortified L-15 medium containing 0.1 μM-dexamethasone, 0.1% BSA and 10% (v/v) fetal-bovine serum. In protein degradation studies, [³H]valine was included during the overnight culture period.

Procedure for isolation of adult hepatocytes

Hepatocytes were isolated from adult animals by perfusion with collagenase as described previously [18]. The final cell pellet was resuspended in fortified L-15 medium, and the cells were plated and treated in a manner identical with that for the cultured fetal cells described above.

Determination of rates of endogenous protein degradation

This was done by a method similar to that described by Mortimore & Mondon [19] and Seglen *et al.* [20]. Liver protein was first pre-labelled by incubating cells with 2.5 μCi of [³H]valine in 2 ml of fortified L-15 medium during the 16 h overnight culture period, as described above. Flasks were washed with 3 × 2 ml of fortified L-15 medium containing 5 mM-valine and then incubated with 2 ml of the same medium for 2 h to allow labelled short-half-life proteins to decay. Cells were then washed with 3 × 2 ml of EH + 3 buffer (Earle's/Hepes supplemented with Ca²⁺, Mg²⁺ and BSA, as described previously [12]) containing 5 mM-valine, and the experimental period was begun (zero time) by addition of 1.0 ml of the same buffer. Inhibitors, hormones and amino acids were added as small portions of concentrated stock solutions which were dissolved or diluted to the desired concentration just before use. Amino acids were added as multiples of the concentrations found in normal rat plasma [21]. Cells were incubated on a rocking platform at 37 °C, and samples of the incubation media were withdrawn at 30 min intervals. Protein was precipitated by addition of trichloroacetic acid (final concn. 10%, w/v). After centrifugation (1 min at 8000 g), a portion of the supernatant fluid was taken for determination of acid-soluble radioactivity by liquid-scintillation spectrometry. One group of triplicate flasks was used for the determination of total radioactivity in protein at zero time. Trichloroacetic acid (10%) was added to each zero-time flask and the contents of the flask were removed by scraping. After centrifugation, a sample of the supernatant fluid was removed for radioactivity counting,

and the pellet was dissolved in 0.1 M-NaOH by heating in a boiling-water bath for 20 min. A portion of the dissolved protein was then counted for radioactivity. The percentage acid-soluble radioactivity was calculated relative to the radioactivity present in total protein at zero time. Results were corrected for the small amount (less than 1% of the total) of acid-soluble radioactivity present in the cells at the start of the experiment.

Extraction of cellular insulin-degradation products from fetal hepatocytes and analysis by h.p.l.c.

Cultured fetal hepatocytes were incubated with a tracer concentration (90 pM) of ¹²⁵I-insulin for 2 h at 37 °C. The incubation medium was removed and the cells were washed with 5 × 2 ml of ice-cold EH + 3 buffer. Then 1 ml of a solution consisting of 7 M-urea, 3 M-acetic acid, 0.2% Triton X-100 and 100 μg of carrier pig insulin/ml was added to each flask, and the contents of the flask were removed by scraping. The pooled extracts from 30 flasks were subjected to gel-filtration chromatography and were analysed by reversed-phase h.p.l.c. as described previously [22,23].

Determination of insulin binding and degradation

Methods for the determination of ¹²⁵I-insulin binding and degradation with cultured fetal hepatocytes [12] have previously been described. Determination of binding and degradation with cultured adult cells was performed exactly as described for the cultured fetal cells.

Determination of internalization rate constants

The ratio of internalized to surface-bound insulin and the internalization rate constant (K_e) were determined as described by Wiley & Cunningham [24]. After plating and attachment, cultured fetal or adult hepatocytes were washed with 2 × 2 ml of EH + 3 buffer. Duplicate flasks were incubated at 37 °C in 1.5 ml of the same buffer and the experiment was initiated by the addition of tracer ¹²⁵I-insulin (final concn. 20 pM) or tracer plus unlabelled insulin (final concn. 2 μM). At the indicated times, cells were quickly washed with 5 × 2 ml of ice-cold EH + 3 buffer. Any remaining fluid was aspirated, and 1.5 ml of ice-cold 0.075 M-acetic acid in 0.19 M-NaCl was added to each flask and the flasks were placed on ice for 5 min. The acetic acid wash was aspirated and kept, and the cell layer was washed with an additional 0.5 ml of the same mixture. The amount of ¹²⁵I-insulin in the combined acetic acid washes, which represents the surface-bound fraction of cell-associated hormone, was determined by γ-ray scintillation spectrometry. The cell layer was treated overnight with 1.0 ml of 1.0 M-NaOH at ambient temperature to solubilize the cells. The extracts were aspirated, and the flasks washed with 2 × 0.5 ml of water. These washes were combined with the NaOH extract and counted for radioactivity. Radioactivity present in this fraction represents internalized ¹²⁵I-insulin. The internalization rate constant was determined from the initial slope of the ratio of internalized to surface-bound hormone versus time [24].

Statistical analyses

Results are presented as means ± S.E.M. for the indicated numbers of experiments in each group. When two sample means were compared, Student's *t* test was employed. When more than two sample means were to be compared, data were analysed by ANOVA followed by Student–Newman–Keuls multiple-range test.

RESULTS

In initial experiments we compared the time course of protein degradation in cultured fetal and adult hepatocytes. As described

in the Materials and methods section, endogenous protein was pre-labelled overnight by incubation of the cells with [^3H]valine, and cells were washed free of unincorporated label, and were

Fig. 1. Time course of protein degradation in cultured fetal (○) and adult (●) hepatocytes

Cell protein was pre-labelled by incubating cells overnight in the presence of [^3H]valine. Protein degradation was then determined based on the release of acid-soluble radioactivity as described in the Materials and methods section. The release of acid-soluble radioactivity is expressed as a percentage of radioactivity present in total protein at zero time.

Table 1. Effect of various agents on endogenous protein degradation in adult and fetal hepatocytes

As described in detail in the Materials and methods section, cells were incubated overnight with [^3H]valine. At the time of the experiment, cells were washed to remove unincorporated label and were preincubated for 2 h to allow short-half-life proteins to decay. The cells were briefly washed again and incubated in a simple salts buffer containing 5 mM-valine (unlabelled) and the indicated additions. Cells were incubated for 2.5 h, at which time samples of the media were withdrawn for the determination of acid-soluble radioactivity. Results are expressed as the percentage of acid-soluble radioactivity released/h, relative to the total radioactivity present at zero time. Numbers in parentheses represent the numbers of individual experiments per group. ^a Significantly different from Basal ($P < 0.05$); ^b significantly different from the No additions group ($P < 0.05$).

	Protein degradation (% of total protein degraded/h)	
	Adult	Fetal
Basal (0.1 μM -insulin + 5 $\times$ plasma amino acids)	2.46 $\pm$ 0.06 ^b (6)	2.48 $\pm$ 0.16 ^b (5)
No additions	4.21 $\pm$ 0.15 ^a (16)	2.90 $\pm$ 0.08 ^a (26)
5 $\times$ plasma amino acids	2.63 $\pm$ 0.10 ^b (15)	2.35 $\pm$ 0.07 ^b (23)
Insulin (0.1 μM)	3.61 $\pm$ 0.13 ^{a,b} (9)	3.05 $\pm$ 0.15 ^a (7)
Dibutyl cyclic AMP (1 mM)	5.23 $\pm$ 0.18 ^{a,b} (4)	3.67 $\pm$ 0.09 ^{a,b} (5)

Fig. 2. Dose-response effect of chloroquine to inhibit protein degradation in cultured fetal hepatocytes

Rates of protein degradation were determined during a 2.5 h incubation of cells in unsupplemented media (without insulin or amino acids) as described in Table 1. The indicated doses of chloroquine were added to the cells at zero time. Results shown are means $\pm$ S.E.M. of 3–6 experiments.

preincubated to allow rapidly turning-over proteins to decay. The cells were washed again, and then incubated in a simple salts buffer. As shown in Fig. 1, in both fetal and adult cells the release of acid-soluble radioactivity was linear for up to 3 h of incubation. All subsequent experiments were therefore terminated after 2.5 h of incubation.

Table 1 summarizes the results of experiments in which a number of agents known to affect total protein degradation in adult hepatocytes were examined in the fetal hepatocyte system. As indicated, the rate of basal protein degradation (that observed in the presence of both insulin and amino acids) was similar in both adult and fetal hepatocytes (2.46 and 2.48 % degraded/h respectively). A deprivation-induced increase in protein degradation was seen in both adult and fetal hepatocytes when they were incubated in buffer alone ('No additions'). However, the increase in adult hepatocytes was significantly greater than that in fetal cells. To ascertain the individual roles of amino acids and insulin in regulating degradation, cells were incubated in the presence of either agent alone. Compared with the no-additions group, a mixture of amino acids at 5 times (5 $\times$) their normal rat plasma concentrations fully suppressed degradation to the basal level in both adult and fetal hepatocytes. Expressed as a

Fig. 3. Dose-response effect of NH_4Cl to inhibit protein degradation in cultured fetal hepatocytes

Experiments were conducted as described in the legend to Fig. 2. Results shown are means $\pm$ S.E.M. of 3–6 experiments.

percentage of the deprivation-induced no-additions group, $5 \times$ amino acids produced a 38 % and 19 % inhibition of degradation in adult and fetal hepatocytes respectively. In preliminary experiments in which insulin concentrations ranging from 10 pM to 1 μ M were tested for their effect on protein degradation in cultured adult hepatocytes, a concentration of 0.1 μ M was shown to be maximally effective (results not shown). As shown in Table 1, 0.1 μ M-insulin produced a significant inhibition of degradation in adult hepatocytes as compared with the no-additions group, although insulin alone was not as effective as the combination of amino acids and insulin together. In contrast, 0.1 μ M-insulin alone had no effect on protein degradation in fetal cells as compared with the no-additions group. The absence of an effect of insulin on protein degradation was consistently seen over a range of insulin concentrations from 10 pM to 1 μ M (results not shown). In both fetal and adult cells, addition of dibutyl cyclic AMP resulted in a significant stimulation of degradation above that observed in the no-additions group (Table 1). A similar stimulation of degradation could also be produced in both groups by addition of 10 nM-glucagon (results not shown).

Other agents or conditions known to influence the degradation of long-half-life proteins in adult hepatocytes were also examined in the fetal hepatocyte system. Chloroquine is a well-known acidotropic agent which produces its effect, at least in part, by raising intralysosomal pH. The dose-response effect of chloroquine to inhibit protein degradation in cultured fetal hepatocytes is shown in Fig. 2. At the highest dose tested (2.5 mM), chloroquine produced a similar inhibition of protein degradation in fetal cells (20–25 %) to that observed with $5 \times$ amino acids. A second lysosomal inhibitor, NH_4Cl , was not as effective as chloroquine, as it inhibited degradation only by about 15–20 % (Fig. 3).

In adult liver, amino acids are considered to be important regulators of proteolysis [25]. A dose-response curve showing the effect of amino acids to inhibit degradation in cultured fetal hepatocytes is presented in Fig. 4. Progressively greater inhibition was observed as the amino acid concentration was increased from zero to $5 \times$ the normal rat plasma concentration, reaching a maximal inhibition of approx. 30 %. No further inhibition was seen as the amino acid concentration was increased from $5 \times$ to $20 \times$. In adult liver, the capacity of amino acids to inhibit protein degradation has been shown to reside in a limited number of amino acids [26,27]. As shown in Fig. 4, when added at $20 \times$ normal plasma concentrations, the seven regulatory amino acids

Fig. 4. Dose-response effect of amino acids to inhibit protein degradation in cultured fetal hepatocytes

Experiments were conducted as described in the legend to Fig. 2. Amino acids were added in multiples ($\times$) of their concentrations found in normal rat plasma [21]: $\bullet$, all 20 amino acids; $\circ$, seven regulatory amino acids [27].

Fig. 5. Dose-response effect of dinitrophenol to inhibit protein degradation in cultured fetal hepatocytes

Experiments were conducted as described in the legend to Fig. 2.

described by Pösö *et al.* [27] also inhibited degradation in our fetal hepatocyte system, but were slightly less effective than was the full mixture of amino acids at the same concentration.

In the adult liver, a significant proportion of both lysosomal and non-lysosomal protein degradation has been shown to be energy-dependent [20]. To determine if energy is also required for protein degradation in fetal hepatocytes, cells were incubated in the presence of various concentrations of dinitrophenol. As shown in Fig. 5, the addition of dinitrophenol at concentrations of 500 μ M and above resulted in a significant inhibition of protein degradation. At the highest concentration, degradation was suppressed to 50–55 % of the control no-additions group.

Further studies were conducted to explore the lack of effect of insulin to suppress accelerated proteolysis in the fetal hepatocyte system. Previous reports suggested that insulin internalization

Fig. 6. H.p.l.c. analysis of insulin-degradation products in cultured fetal hepatocytes

Cells were incubated at 37 °C for 2 h with 90 pM- ^{125}I -insulin in Leibovitz L-15 medium with 1 % BSA. The medium was discarded, and the cells were washed and extracted as described in the Materials and methods section. The resulting samples were chromatographed on Sephadex G-50, and the insulin-sized fractions were pooled, freeze-dried and analysed by reversed-phase h.p.l.c. [22,23].

Fig. 7. Dose-response effect of chloroquine on ^{125}I -insulin binding and degradation in cultured fetal (Δ) and adult (●) hepatocytes

Cells were incubated with 40 pM- ^{125}I -insulin and the indicated concentration of chloroquine for 2 h at 37 °C. Insulin binding (cell-associated radioactivity) and degradation (trichloroacetic acid-soluble products) were then determined as described in the Materials and methods section. Results are expressed as a percentage of the binding (a) or degradation (b) observed in the no-inhibitor control and are means $\pm$ S.E.M. from 3–5 experiments.

and processing may be a necessary component of the hormone's effect to inhibit protein degradation in liver [18,28]. We therefore examined insulin processing in our cultured fetal hepatocyte system. Fetal hepatocytes were incubated with specifically labelled ^{125}I (A14)-insulin for 2 h at 37 °C. The insulin was then extracted from the cells, the extract was applied to a gel-filtration column, and the insulin-sized material eluted from the column was pooled, freeze-dried, and applied to a reversed-phase h.p.l.c. column. As shown in Fig. 6, most of the radioactivity was eluted from the h.p.l.c. column at 51 min, in the same position as the intact ^{125}I -insulin. Similar to what has previously been reported for adult hepatocytes [22,23] and the perfused kidney [29], a characteristic doublet consisting of two insulin-degradation products less hydrophobic than insulin was eluted from the h.p.l.c. column, at 19 and 23 min. Thus it would appear that the initial degradation products of insulin are identical in fetal and adult hepatocytes.

Chloroquine has provided a useful tool to aid in the understanding of the processes of insulin uptake and degradation by cells. In addition to inhibiting lysosomal function, and thus the final stages of insulin degradation, chloroquine is also thought to produce its effects on insulin processing, at least in part, by inhibiting endosomal acidification and the subsequent fusion of

Fig. 8. Determination of internalization rate constants in cultured fetal (●) and adult (○) hepatocytes

Cells were incubated at 37 °C with 30 pM- ^{125}I -insulin for the indicated times. Cells were then rapidly washed with ice-cold buffer and extracted for 5 min at 0 °C with 0.075 M-acetic acid in 0.19 M-NaCl to remove surface-bound radioactivity. Cells were then hydrolysed in 1 M-NaOH to determine internalized radioactivity. The ratio of internalized to surface-bound radioactivity is plotted versus time. The internalization rate constant (K_i) was determined by linear-regression analysis of the initial slope (0–12 min) in each case.

endosomes with lysosomes [30]. If differences in uptake and degradation of insulin exist between fetal and adult cells, we reasoned that these differences might be manifested as differences in sensitivity to chloroquine. Cultured fetal or adult hepatocytes were therefore incubated at 37 °C with ^{125}I -insulin and various doses of chloroquine for 2 h. Samples were then processed for determination of insulin binding (total cell-associated ^{125}I -insulin) and insulin degradation. As shown in Fig. 7, chloroquine enhanced insulin binding and decreased insulin degradation in both adult and fetal hepatocytes. The fetal system showed a somewhat greater response to chloroquine, however, as at higher doses of chloroquine binding was enhanced and degradation was inhibited to a greater extent in fetal than in adult hepatocytes.

Differences in insulin internalization and degradation between fetal and adult cells could be of either a qualitative or quantitative nature, or both. Since the results of the h.p.l.c. analyses and the chloroquine studies suggested that qualitatively similar mechanisms for insulin processing and degradation are present in both fetal and adult hepatocytes, we next compared the rate of uptake of insulin in both groups. The internalization rate was determined from the approach to steady state of the ratio of internalized to surface-bound insulin, as described in the Materials and methods section. From the results shown in Fig. 8, the rate constant for internalization (K_i) of insulin in adult cells was calculated to be $0.185 \pm 0.012 \text{ min}^{-1}$. The comparable value in fetal cells ($0.129 \pm 0.031 \text{ min}^{-1}$) was approx. 30 % lower than that in adult cells ($P < 0.05$).

DISCUSSION

Although numerous studies have examined factors influencing overall fetal growth and the growth of specific organs in the fetus (see [31] for review), no definitive studies on the regulation of protein turnover in the fetal liver have been conducted to date. A major complicating factor in studies of hepatic protein turnover during fetal development is the presence of haematopoietic cells in fetal livers. Haematopoietic cells are abundant in fetal livers and comprise nearly half of the liver volume shortly before birth [14,15]. Estimates of rates of haemoglobin synthesis indicate that as much as 20 % of the protein-synthetic activity in the fetal liver occurs in haematopoietic cells, rather than in hepatic

parenchymal cells [32]. As a result, interpretation of isotopic studies of protein turnover *in vivo* in fetal liver can be greatly complicated by the presence of haematopoietic cells. The ability to conduct studies free of the potential source of error that haematopoietic cells could introduce is essential to gaining an understanding of protein turnover in fetal hepatocytes, and is a significant feature of our experimental system.

During the final 3 days before birth in the rat, the liver undergoes a striking acceleration of growth [14,15,33,34]. During this time, glycogen deposition increases dramatically [14,15,33], the volume of individual parenchymal cells triples [14,15], and the total protein content of the liver increases 2-fold [33,34]. Whether changes in protein synthesis, protein degradation or both are responsible for the rapid growth and increase in hepatic protein content is largely unknown. Apparent rates of protein synthesis were higher in homogenates and subcellular fractions [35] or in slices [36] from fetal-rat livers as compared with adults. Estimates of protein synthesis in livers of fetal lambs were 10 times higher than comparable measurements in adult livers [37]. Goldspink & Kelly [34] calculated a rate of protein degradation based on measured rates of protein synthesis and net changes in liver protein content. They reported that, at 16 days of fetal development, the calculated rate of protein degradation was similar to that in adult animals, but rose at 18 and 20 days of fetal life to a value approximately twice that in adults [34]. Based on their studies, one would conclude that the increase in hepatic protein content just before term in the rat is largely the result of a marked increase in protein synthesis. However, as indicated above, the presence of haematopoietic cells greatly confounds the interpretation of these results. In one study in which the haematopoietic contribution was taken into account, the rate of peptide-chain elongation in fetal hepatic parenchymal cells was estimated to be only 30 % of that in livers of adult animals [32].

The majority (98–99 %) of resident liver proteins are considered to be 'long-half-life proteins', whose degradation is regulated by such factors as nutrient availability and hormones [25]. The 'short-half-life proteins', which comprise the remaining 1–2 % of liver proteins, are of considerably lesser importance with regard to liver protein accretion and liver growth. However, because of their very rapid turnover, the short-half-life proteins can greatly influence radioisotopic determinations of protein degradation if appropriate experimental designs are not employed. In our experiments, total liver protein was first pre-labelled overnight. A 2 h preincubation period allowed for significant decay of labelled short-half-life proteins and for release of labelled secretory proteins before the start of the experimental period. The essentially linear release of acid-soluble radioactivity which we observed (Fig. 1) is consistent with the notion that we were predominantly measuring the decay of long-half-life proteins.

As shown in Table 1, the basal rate of proteolysis was similar in fetal and adult hepatocytes. These rates are also similar to what has previously been reported in freshly isolated and cultured hepatocytes [26,38], which typically show a slightly higher rate of basal proteolysis than is seen in a perfused liver system [27,39]. In adult hepatocytes, the deprivation-induced acceleration of proteolysis reached a similar magnitude to that seen in perfused livers [39], but in fetal cells the acceleration was far less. The reason for this difference is not known. An increase in macroautophagy is generally considered to be responsible for the acceleration of proteolysis under step-down or deprivation conditions [39]. To the best of our knowledge, no morphometric evidence of macroautophagy in the fetal liver has been presented. Rohr *et al.* [14] found large increases in the volume density of autophagic vacuoles in livers of animals 24 h after birth. The vacuoles were seen to contain mitochondria and fragments of the rough endoplasmic reticulum, similar to observations made

during periods of nutrient deprivation in adult-rat livers [40]. Lysosome-like structures have been noted in fetal-rat livers 3 days before birth [41], but whether they are functional remains to be determined.

In both adult and fetal hepatocytes, accelerated proteolysis could be suppressed by the addition of a mixture of amino acids at 5 times their normal rat plasma concentrations. Addition of dibutyl cyclic AMP significantly stimulated proteolysis above that seen in the unsupplemented state in both the adult and fetal cells. Although the fetal liver has previously been reported to be somewhat unresponsive to glucagon [42], we were able to produce effects similar in magnitude to those seen with cyclic AMP by addition of 10 nM-glucagon to the fetal cells (results not shown). The effect of insulin to inhibit hepatic protein degradation in adult liver is well known [19,43], and was readily evident in our cultured adult cells. Surprisingly, however, insulin was totally without effect on protein degradation in cultured fetal cells (see Table 1). Insulin receptors are present in liver at this stage of development [4–8], and we [12] and others [44–46] previously demonstrated an increased rate of glucose incorporation into glycogen in response to insulin in fetal hepatocytes. Also unlike the situation in adult liver, insulin is without effect on amino acid transport in fetal liver cells as well [11,12]. It therefore appears that the fetal liver displays a partial resistance to some of the actions of insulin, but not others. We also tested a number of other peptide hormones and growth factors, including epidermal growth factor, insulin-like growth factor-II, growth hormone and prolactin, and none of these had any effect on accelerated proteolysis as well (results not shown).

Given the effect of amino acids, chloroquine and NH_4Cl to inhibit protein degradation in fetal hepatocytes, it was somewhat surprising that insulin was without effect in these cells. However, these results suggested that the lack of effect of insulin may be the result of an impairment or immaturity in the insulin signal-transduction process, rather than being due to a general impairment in some component of the protein-degradation machinery in fetal livers. Previous reports presented evidence consistent with the notion that the internalization and processing of insulin are necessary for the hormone's effect to inhibit protein degradation in adult cells [18,28]. Incubation of adult cells with dansylcadaverine, which appears to inhibit insulin processing at a step after internalization but before the chloroquine-sensitive step [18], eliminated the effect of insulin to inhibit protein degradation [18,28]. Of interest, insulin had no effect on amino acid transport in dansylcadaverine-treated adult cells, but did stimulate glycogen synthesis under these conditions [18]. The dansylcadaverine-treated adult hepatocyte and the fetal hepatocyte therefore display a similar pattern of partial insulin resistance [12], and might therefore be expected to display an impairment in insulin processing at the same or similar steps. For these reasons, the characterization of insulin processing in fetal hepatocytes was of interest. Examination of insulin-degradation products in fetal hepatocytes (Fig. 6) revealed a profile which was identical with that in untreated adult hepatocytes [22,23]. Incubation of adult cells with dansylcadaverine markedly alters the pattern of insulin-degradation products [23]. Although these results do not entirely rule out the possibility, it seems unlikely that fetal hepatocytes and dansylcadaverine-treated adult hepatocytes exhibit identical impairments in insulin processing.

Chloroquine has been extensively utilized in studies of insulin processing and insulin degradation. In addition to its effect to inhibit lysosomal function, it appears that chloroquine primarily inhibits insulin processing and degradation at a pre-lysosomal step, resulting in the accumulation of insulin in endosomes [30]. As in adult cells, incubation of fetal cells with chloroquine resulted in an increase in cell-associated insulin, and a decrease

in the generation of trichloroacetic acid-soluble insulin-degradation products (Fig. 7). Chloroquine produced a somewhat larger effect on both insulin binding and degradation in the fetal cells as compared with the adult, suggesting that a greater proportion of insulin degradation in fetal hepatocytes occurs via a chloroquine-sensitive pathway.

The results of the studies described above suggested qualitatively similar mechanisms for insulin processing in fetal and adult hepatocytes. To determine whether quantitative differences in insulin uptake might account for the differences observed, rates of insulin internalization were compared in cells from fetal and adult livers. As indicated in Fig. 8, fetal cells exhibited a 30% slower rate of insulin internalization as compared with adult cells. Although it must remain a possibility, it seems unlikely that this small decrease in the internalization rate would be responsible for the complete absence of an insulin effect on protein degradation.

As our results show, insulin has no acute effect on protein degradation in the fetal hepatocyte. However, our results do not rule out the possibility that chronic exposure of the cells to insulin is required before an effect on protein degradation is seen, or that insulin has a permissive role to allow the regulation of protein degradation by other growth factors. Further studies will be required to elucidate the mechanisms responsible for the partial insulin resistance in fetal liver.

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