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# Apolipoprotein Synthesis in Newborn Piglet Intestinal Explants

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**ABSTRACT. To determine the effects of hormones and epidermal growth factor (EGF) on the small intestinal synthesis of apolipoproteins B-48, A-I, and A-IV in the neonatal mammal, apolipoprotein synthesis by proximal jejunal explants from 2-d-old female piglets was studied in tissue culture. Initial comparison studies with various media showed optimal total protein and apo A-I synthesis with Williams' medium E without fetal bovine serum. Sets of explants were prepared containing EGF and various hormones in the medium. After $^{35}$S-methionine radiolabeling, explants were homogenized, and specific apolipoprotein synthesis was quantitated by immunoprecipitation as the percentage of total protein synthesis. Apo B-48 synthesis was not affected by any additives except the combination of EGF and hydrocortisone, which slightly decreased synthesis. Apo A-I synthesis was significantly increased by EGF. This EGF-induced increase in apo A-I synthesis was blunted by concomitant treatment with hydrocortisone. In contrast, the combination of insulin and hydrocortisone induced a significant increase in apo A-I synthesis. Although EGF and insulin modestly increased apo A-IV synthesis, the combination of insulin and hydrocortisone treatment up-regulated apo A-IV synthesis by 2.6-fold. Thyroid hormone lacked effect on synthesis of any of the apolipoproteins. EGF, glucocorticoids, and insulin may play regulatory roles in the developmental expression of apolipoprotein synthesis in the neonatal small intestine. (*Pediatr Res* 32: 553–558, 1992)**

**Abbreviations**

**EGF, epidermal growth factor**
**FBS, fetal bovine serum**

Apolipoproteins are surface components of lipoprotein particles and serve essential functions in the secretion, metabolism, and receptor-mediated uptake of these particles. Apo B is present in triglyceride-rich lipoproteins, and there are two distinct forms of apo B in the human, rat, and swine (1–3). Apo B-100 is the larger form and is a component of plasma VLDL and LDL and contains the LDL receptor binding domain (4). Apo B-48 is the smaller species found in intestinal chylomicrons and does not contain the LDL receptor binding domain (5). In the postnatal human and swine, apo B-100 and apo B-48 are predominately of hepatic and intestinal origin, respectively (1, 6). In both liver and intestine, apo B is thought to be essential for the packaging and secretion of triglyceride-rich lipoproteins (3).

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Apo A-I is the major apolipoprotein of plasma HDL and is produced by both liver and intestine in the human, rat, and swine (1, 7–10). Its major metabolic role is that of a cofactor for lecithin:cholesterol acyltransferase, the enzyme responsible for the production of most plasma cholesteryl esters (11). Apo A-IV is a component of nascent intestinal lipoproteins, including chylomicrons and HDL (12–15), and becomes dissociated from chylomicrons soon after secretion (16, 17). The major metabolic function of apo A-IV is presently unknown. Recent *in vitro* studies have suggested roles in the activation of lecithin:cholesterol acyltransferase (18, 19) and in reverse cholesterol transport by promoting cellular cholesterol efflux (20) and serving as a ligand for HDL binding to hepatocytes (21).

The small intestine plays a key role in neonatal lipid metabolism because of the importance of dietary lipid as a nutrient source during this period. Potential factors responsible for the developmental regulation of small intestinal function include diet, the microenvironment of the enterocyte (hormones, growth factors, and the extracellular matrix), and preprogrammed genetic cues. Because apolipoproteins are crucial to the biogenesis and peripheral metabolism of lipoproteins, regulation of their expression in the developing gut is of obvious importance. Studies of the developmental regulation of intestinal lipoprotein metabolism have been largely performed in the rat (22–26), an altricial species as compared with the precocial human. We have developed a swine model for the study of intestinal apolipoprotein gene expression in the developing mammal (1, 27–29). The swine is a precocial mammal and has significant homology with the human with regard to lipoprotein metabolism (1, 7, 27, 28) and intestinal development in general (30). We have so far shown distinct regulatory patterns for intestinal expression of apo B, apo A-I, and apo A-IV induced by dietary and biliary lipid in the neonatal and older suckling piglet using an *in vivo* model (1, 27–29). To study the potential regulatory roles of hormones and growth factors, a system for the short-term culture of newborn piglet intestinal explants in serum-free medium was developed. This system offers the advantages of control over the microenvironment of the enterocytes while maintaining the native extracellular matrix. In the present report, the effects of hydrocortisone, insulin, thyroid hormone, and EGF on apolipoprotein synthesis in newborn piglet jejunal explants were studied.

## MATERIALS AND METHODS

*Animals.* Two-d-old female domestic swine were obtained from Research Industries Corporation (Monee, IL).

*Jejunal explant preparation.* From the time of arrival to the time of surgery the next day, the 2-d-old animals were kept in heated isolettes. Animals were fed artificial sow's milk (SPF-LAC, Pet-Ag, Inc., Hampshire, IL) by gavage during this period. Anesthesia was induced by intramuscular ketamine at a dose of 0.15 mmol/kg (40 mg/kg) and maintained by face mask delivering 1 L/min $O_2$ and 0.5–0.8% halothane. A longitudinal midline abdominal incision was made, and the peritoneal cavity was

opened. Starting 10 cm distal to the ligament of Treitz, a 20-cm segment of proximal jejunum was isolated with ligatures and removed. The lumen of the segment was then rinsed with PBS followed by Earle's balanced salt solution. The segment was placed in Earle's balanced salt solution for transport to the laboratory. The segment was then opened longitudinally, and 2 × 2 mm explants were prepared with the serosa and muscularis mucosae intact. Explants were then placed on nylon grids in Falcon tissue culture dishes (Becton Dickinson and Co., Lincoln Park, NJ) (six explants per dish) (31). Explants for the first part of the study, determining optimal culture conditions, were derived from three piglets. All explants for the portion of the study examining the effects of EGF and hormones were derived from two animals contributing explants to all experimental groups.

*Explant culture.* Initial studies were designed to identify the optimum culture medium for the explants to support apolipoprotein synthesis. A variety of culture media were tested including minimum essential medium of Eagle, Dulbecco's modified Eagle medium, cell suspension modified Eagle medium, Williams' medium E, Leibovitz's medium L-15, and RPMI 1640 medium with and without FBS at a concentration of 10%. In these initial studies, explants were cultured for 18 h in a humidified incubator at 37°C in 5% $CO_2$. Then 15 $\mu$Ci $^{35}$S-methionine (Amersham, Arlington Heights, IL) in fresh medium were added to each culture dish for a 6-h incubation for a total culture time of 24 h. All media contained 20 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 2 mmol/L, glutamine, and antibiotics.

After optimization of basal culture conditions, explant cultures were carried out with the addition of the following hormones and growth factors to the medium: EGF at 82.6 nmol/L (500 ng/mL), insulin at 43.6 pmol/L (6 mU/mL), hydrocortisone at 138 nmol/L (50 ng/mL), and thyroxine at 64.4 pmol/L (0.05 ng/mL). Besides studying the effects of these additives singly, the following combinations were studied at the same individual concentrations: insulin plus hydrocortisone and EGF plus hydrocortisone. These additives were present in the culture media during the entire culture period with radiolabeling carried out during the final 6 h of culture.

*Apolipoprotein immunoprecipitation.* After incubation, explants were rinsed in cold PBS and homogenized in PBS containing 20 mmol/L unlabeled methionine, 1% Triton X100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, and 1 mmol/L leupeptin, pH 7.4. Aliquots of the homogenate were taken for measurement of total protein concentration and trichloroacetic acid-precipitable radioactivity. The remainder was pelleted at 105 000 × $g$ for 60 min in a 50.3 titanium rotor (Beckman Instruments, Palo Alto, CA), followed by collection of the supernatant. Although most intracellular apolipoprotein is membrane-associated, this technique has been shown previously to result in extraction and solubilization of 84–94% of total recoverable apolipoprotein mass (32). All procedures were performed at 0–5°C, and the supernatant samples were stored at −80°C until analysis. Intestinal supernatant fractions were subjected to specific immunoprecipitation of apo B, apo A-I, and apo A-IV under conditions of antibody excess as described (27, 28). Aliquots of supernatants were mixed with washed IgG-Sorb (The Enzyme Center, Malden, MA) and subsequently reacted with excess anti-apolipoprotein antiserum for 18 h at 4°C. After a second addition of IgG-Sorb and extensive washing, the liberated immunocomplex was applied to either 5.6% (apo A-I and apo A-IV) or 4% (apo B) SDS polyacrylamide tube gels (27, 28). After electrophoresis, gels were sliced into 1-mm slices and incubated in 3% Protosol/97% Econofluor (New England Nuclear, Boston, MA) at 37°C overnight before liquid scintillation counting in a Packard Model 2000 liquid scintillation counter (Packard Instruments, Downers Grove, IL). Apolipoprotein species were identified by comparison to stained coelectrophoresed apolipoproteins. Apolipoprotein synthesis rates were expressed as the percentage of specific immunoprecipitated apoprotein counts compared with total protein trichloroacetic acid-precipitable counts. Apolipoprotein synthesis was thereby expressed as a percentage of total protein synthesis. All samples were subjected to reimmunoprecipitation to ensure the completeness of the first antigen-antibody reaction. Total protein content of the explant homogenates was measured by a modified Lowry technique (33).

*Statistical analysis.* Data in experimental groups (control and treatment with hormones and EGF) were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test to compare specific groups. The null hypothesis was rejected at $p < 0.05$.

## RESULTS

*Total protein and apo A-I synthesis with various culture media with and without FBS.* Figures 1 and 2 show total protein sp act and apo A-I synthesis, respectively, with explants cultured in various media with and without FBS. Each bar represents the means of data from two culture dishes containing six explants

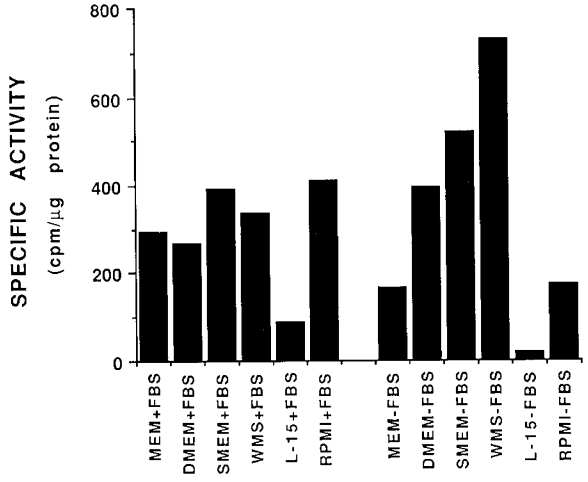


Fig. 1. Homogenate sp act from jejunal explants cultured in various media. Media tested include minimum essential medium of Eagle (*MEM*), Dulbecco's modified Eagle medium (*DMEM*), cell suspension modified eagle medium (*SMEM*), Williams' medium E (*WMS*), Leibovitz's medium L-15 (*L-15*), and RPMI 1640 medium (RPMI) with and without FBS (± FBS, 10%). *Bars* represent the mean of data from two culture dishes of explants (6 explants/dish).

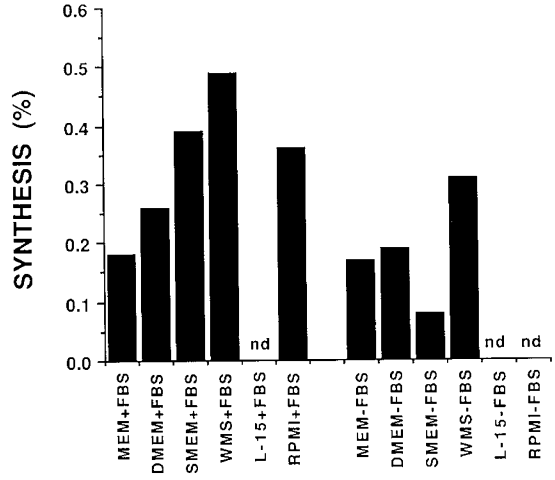


Fig. 2. Apo A-I synthesis in jejunal explants cultured in various media with and without FBS. Abbreviations for the various culture media are the same as in Figure 1. *Bars* represent the mean of data from two culture dishes of explants (6 explants/dish). nd, none detectable.

per dish. Williams' medium E without FBS clearly supported the highest explant homogenate sp act. Although apo A-I synthesis (Fig. 2) in this medium was somewhat higher with the addition of FBS than without, this medium yielded the highest apo A-I synthesis when compared with the other media with or without FBS supplementation. Because we preferred to use serum-free medium in our studies with hormone and growth factor supplementation, Williams' medium E was used in all subsequent experiments. Additionally, in subsequent studies we shortened the total culture time to 12 h with radiolabeling during the final 6 h, as this resulted in higher basal apo A-I synthesis compared with the longer culture time (1.46 *versus* 0.31% of total protein synthesis).

*Effect of hormones and growth factors on explant total protein and apolipoprotein synthesis.* Data from these studies are depicted in Figures 3-6. Each bar represents data (mean ± SEM) from five culture dishes containing six explants per dish. Figure 3 shows total protein sp act of radiolabeled homogenates from the various experimental groups. A modest, yet significant, increase in radiolabel incorporation is seen with EGF treatment. Yet, a 9-fold increase is induced by treatment with the combination of insulin and hydrocortisone.

Apo A-I synthesis is shown in Figure 4. Paralleling the changes in total protein radiolabeling are modest, but significant, increases in apo A-I synthesis with EGF and the combination of insulin and hydrocortisone. The increase induced by EGF is not present with concomitant treatment with hydrocortisone. Figure 5 shows apo B synthesis. None of the additives had a significant effect except a modest, though statistically significant, decrease in synthesis with the combination of EGF and hydrocortisone. Only apo B-48 synthesis, not apo B-100, was identified in all the samples. Apo A-IV synthesis is shown in Figure 6. Although EGF and insulin individually increase apo A-IV synthesis to a modest degree, the combination of insulin and hydrocortisone treatment up-regulates apo A-IV synthesis by 2.6-fold. Thyroid hormone appears to have no effect on explant synthesis of any of the apolipoproteins studied.

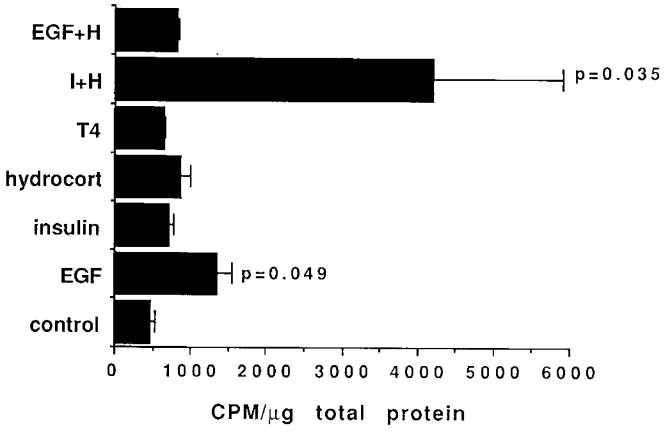


Fig. 3. Homogenate sp act from newborn piglet jejunal explants cultured with EGF, insulin, hydrocortisone (*hydrocort*), thyroid hormone (*T4*), insulin plus hydrocortisone (*I+H*), and EGF plus hydrocortisone (*EGF+H*). Control explant medium contained no additives. *Bars* represent mean ± SEM of data from five culture dishes of explants (6 explants/dish). Statistically significant differences from the control values are indicated by the designated *p* values.

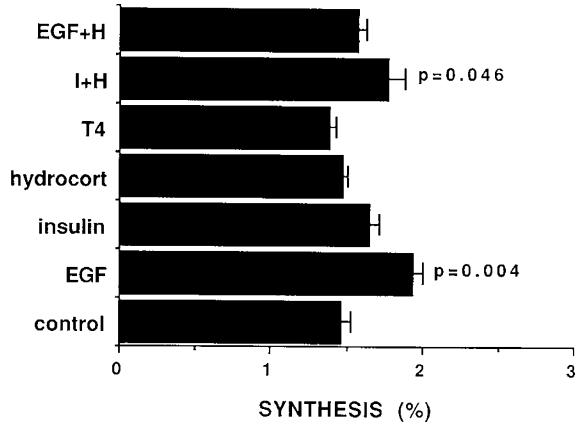


Fig. 4. Apo A-I synthesis as a percentage of total protein synthesis in jejunal explants cultured with hormones and EGF (abbreviations are the same as in Fig. 3). *Bars* represent mean ± SEM of data from five culture dishes of explants (6 explants/dish). Statistically significant differences from the control values are indicated by the designated *p* values.

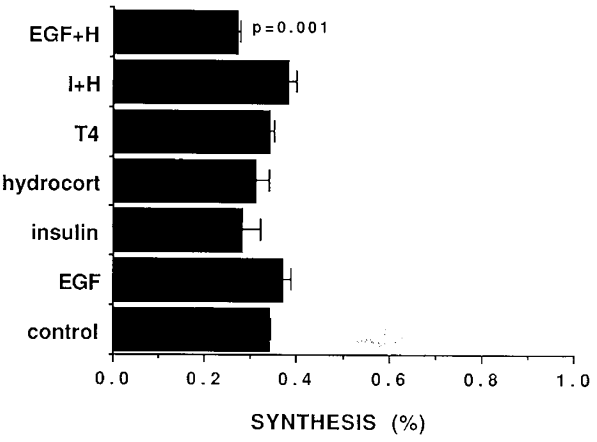


Fig. 5. Apo B synthesis as a percentage of total protein synthesis in jejunal explants cultured with hormones and EGF (abbreviations are the same as in Fig. 3). *Bars* represent mean ± SEM of data from five culture dishes of explants (6 explants/dish). Statistically significant differences from the control values are indicated by the designated *p* values.

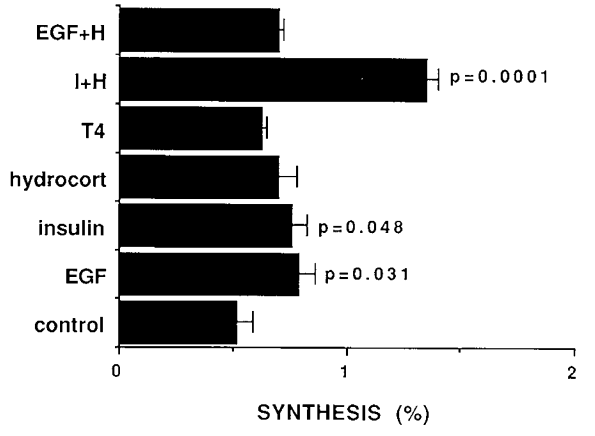


Fig. 6. Apo A-IV synthesis as a percentage of total protein synthesis in jejunal explants cultured with hormones and EGF (abbreviations are the same as in Fig. 3). *Bars* represent mean ± SEM of data from five culture dishes of explants (6 explants/dish). Statistically significant differences from the control values are indicated by the designated *p* values.

## DISCUSSION

The first phase of the present studies involved the testing of several different standard culture media for their suitability in supporting active total protein and apolipoprotein synthesis by jejunal explants from the newborn piglet. Media were tested both in the presence and absence of FBS. Obviously, for our studies of the effects of hormones and growth factors, serum-free culture conditions were preferred. Based on previously reported studies of the serum-free culture of fetal human intestinal explants (34,

35), we expected Leibovitz's medium L-15 to be optimum. In fact, this medium exhibited the worst total protein synthesis of all the media, and no apo A-I synthesis could be detected. Interestingly, a medium developed for the culture of rat hepatocytes, Williams' medium E, proved to be the best medium, particularly under serum-free conditions. Compositional analysis of the media revealed two major differences between these media. First, the L-15 medium contains galactose as the only carbohydrate source, whereas Williams' medium E and the other media contain only glucose, suggesting that the former may not be a suitable carbohydrate substrate for these cells. Second, Williams' medium E was the only medium tested to contain lipid (linoleate as the methyl ester at $10^{-7}$ mol/L), which may be required for *in vitro* apolipoprotein expression. Regardless of the reason, Williams' medium E was clearly the most suitable medium for the study of apolipoprotein synthesis in newborn piglet intestinal explants.

Most work to date involving the developmental biology of the mammalian small intestine has examined the role of hormones and growth factors in the maturation of the rodent small intestine (22, 36–39). Yet, the neonatal rat differs significantly from the human infant. The rat is an altricial species and is relatively immature at birth, with most maturation of the gastrointestinal tract occurring postnatally in close coordination with weaning. In contrast, the human is a precocial species, and many gastrointestinal functions are mature at birth, though the composition of the diet of the newborn does not necessarily require such maturity (40, 41). Intestinal maturation in the rat is also closely coordinated with postnatal surges in hormones, particularly corticosteroids and thyroid hormone, which increase in the plasma just before a reduction in intestinal lactase activity and an increase in sucrase activity (38, 40). These hormones appear to modulate the expression of these disaccharidases, which is initiated by genetically programmed developmental cues (38, 40). The developing swine is in many ways closer to the human infant than the rat with regard to maturation of gastrointestinal function, with early decline in intestinal lactase activity, increase in sucrase activity, and intestinal closure to macromolecule permeability (30, 38). Plasma cortisol levels in the piglet are high at birth and decline to adult levels by 5 d of age (42). In contrast, plasma concentrations of thyroxine and triiodothyronine are maximal at 23 and 16 d of age, respectively, after much gastrointestinal maturation has already occurred (43). Because our previous *in vivo* studies have shown that the magnitude of modulation of apolipoprotein expression by lipid absorption is maximal in the 2-d-old newborn piglet (27, 28), as well as basal apolipoprotein expression compared to other postnatal developmental groups (1, 27, 28), we chose this developmental stage for the present explant studies.

EGF is a small (molecular mass = 6054 D) polypeptide found in several body fluids, including gastrointestinal secretions, breast milk, and amniotic fluid (39, 44). It exerts a trophic effect on epithelia, including the intestinal mucosa, with stimulation of maturation and cellular proliferation (39, 44). EGF binds to a protein kinase membrane receptor that autophosphorylates and sets a phosphorylation cascade into effect, resulting in stimulation of ornithine decarboxylase and DNA synthesis (39). The role of EGF in the ontogeny of mammalian small intestinal function is not precisely defined. *In vivo* and *in vitro* studies to date in pre- and postnatal rodents, as well as human fetal explants, have provided conflicting results with regard to the effects of parenteral and luminal EGF on small intestinal DNA and protein synthesis, as well as brush border disaccharidase activities (36, 37, 39, 44–48). Furthermore, it is not certain at what level EGF may exert some of these effects, because some effects noted in the intact animal are not found in intestinal culture studies, suggesting that the intestinal EGF effects may be part of a systemic response to EGF (45). In the present study, EGF was added to the explant medium at a concentration that approximates the upper limit of the concentration in human colostrum (49). At this concentration, EGF significantly stimulated total protein synthesis in the piglet jejunal explants. In addition, apo A-I and apo A-IV synthesis, expressed as a percentage of total protein synthesis, increased significantly with EGF treatment. The cellular mechanisms of these effects on apolipoprotein synthesis are unknown, but two possibilities exist. The first is induction of a generalized increase in cellular protein synthesis, as reflected by the increase in total protein sp act. However, only synthesis of apo A-I and A-IV was affected, and the increase in apolipoprotein synthesis was proportionately greater than that of total explant protein synthesis, because apolipoprotein synthesis, expressed as a percentage of total protein synthesis, significantly increased. The second possibility is a more specific action of EGF on induction of the apo A-I and A-IV genes, possibly though *trans*-acting transcription regulatory factors, such as has been demonstrated for the increase in transcription of the gastrin gene induced by EGF (50). This mechanism may be further supported by the fact that the apo A-I and A-IV genes are located in a cluster with apo C-III (51). Interestingly, the addition of hydrocortisone to the EGF abolished the EGF-induced increase in total protein, apo A-I, and apo A-IV synthesis. The combination of EGF and hydrocortisone also modestly decreased apo B-48 synthesis. The mechanism of these inhibitory effects of hydrocortisone is unknown at present, but may involve induction of an intermediate that blocks the stimulatory effect of EGF at some level. This inhibitory effect may be teleologically important if the down-regulation of synthesis of these apolipoproteins is advantageous to the organism during times of stress (such as premature weaning) or in the immediate newborn period when piglet serum cortisol levels are extremely high (42).

Hydrocortisone alone lacked effect on either total protein synthesis or apolipoprotein synthesis in piglet jejunal explants, although it was added to the explants at a concentration comparable to that found in the plasma of the newborn piglet (42), as well as the concentration shown to induce lactase, alkaline phosphatase, and DNA synthesis in human fetal small intestinal explants (34). Yet, the combination of hydrocortisone and insulin greatly increased total protein and apo A-IV synthesis and effected a modest increase in apo A-I synthesis. Insulin treatment alone caused a modest increase in apo A-IV synthesis. With regard to the cellular mechanisms of these actions of insulin and hydrocortisone, one may speculate that our observations are due to a global stimulation of cellular protein synthesis, although, as we noted above for the effects of EGF, apo A-I and A-IV synthesis was affected disproportionately to total protein synthesis. In addition, glucocorticoids generally exert a proteolytic effect resulting in overall negative nitrogen balance. Specific induction of the apo A-I and A-IV genes by insulin and hydrocortisone is certainly possible. The human, rat, and mouse apo A-IV genes contain sequences in the 5′ upstream region and exon 1 that are homologous to consensus sequences for glucocorticoid responsive elements (52). The additive action of hydrocortisone with insulin in our studies may be due to the requirement by insulin of an intermediary induced by hydrocortisone for full induction of apolipoprotein gene transcription. Studies are underway to elucidate these issues. In the intact animal, plasma insulin levels vary over a wide range depending upon the glycemic state of the piglet and environmental temperature (53). Also, insulin in breast milk has been shown to affect glucose metabolism in the newborn piglet (54). Therefore, the effect of the interaction of these two hormones may be important in the postprandial period. A similar effect on apo A-IV expression in mature rat hepatocytes by corticosteroid and insulin has been reported by Elshourbagy *et al.* (23). These investigators noted an increase in apo A-IV mRNA abundance in primary cultures of rat hepatocytes incubated with dexamethasone and insulin, both singly and in combination (23). It is possible that a similar regulatory mechanism is present in the newborn piglet small intestine, although we did not observe a significant effect with hydrocor-

tisone alone. Perrin-Ansart *et al.* (25). studied apo A-I synthesis in intestinal explants from fetal and newborn rats. The addition of dexamethasone to the culture medium lacked effect on explant apo A-I synthesis at d 18 of gestation or at postnatal ages 0, 2, and 5 d (25). Yet, apo A-I synthesis was stimulated by dexamethasone at d 20 of gestation. The addition of insulin to the dexamethasone in those studies did not enhance the effect of dexamethasone alone. The lack of glucocorticoid effect on apo A-I synthesis in the newborn rat pup explants agrees with our findings in explants from the newborn piglet. However, we did observe a stimulation of apo A-I synthesis with the combination of insulin and hydrocortisone. We speculate that our results may vary from those obtained in the rat because of species differences.

In the present study, thyroid hormone lacked effect on synthesis of total protein or any of the apolipoproteins. This finding may not be surprising because the plasma level of thyroxine in the piglet peaks at 3 wk of age (43), and enterocytes may not be responsive in the immediate newborn period. However, any conclusions regarding the lack of effect of thyroid hormone in these studies must be cautiously drawn because we did not have a positive control (a parameter positively modulated by thyroid hormone). In addition, we did not eliminate the possibility of thyroid hormone degradation during the course of the experiments and did not assess explant thyroxine uptake, receptor status, or cellular deiodination pathways.

With regard to our methodology for determining apolipoprotein synthesis in the explants, insulin and hydrocortisone are known to have pronounced and different effects on protein metabolism, and in protein radiolabeling studies the intracellular free amino acid pool and sp act reflect the net effects of protein synthesis and breakdown. Therefore, it is possible that our results may partially reflect a change in precursor methionine sp act. However, explants were cultured in Williams' medium E, which contains methionine at 100 $\mu$mol/L concentration, at all times, including the radiolabeling period. Therefore, the extracellular methionine pool probably remained relatively constant, although we did not measure medium methionine levels. The intracellular methionine concentration was not measured either. Although it is unlikely that the explants were intracellularly depleted in the face of provision of extracellular methionine, this possibility cannot be ruled out.

In summary, we have determined optimum conditions for the short-term culture of newborn piglet small intestinal explants to support apolipoprotein synthesis. Apo B-48, A-I, and A-IV were all synthesized by fasting newborn piglet jejunal explants cultured in serum-free medium. Apo B-48 synthesis was not affected by any additives except the combination of EGF and hydrocortisone, which slightly decreased synthesis. Apo A-I synthesis was significantly increased by EGF. This EGF-induced increase in apo A-I synthesis was blunted by concomitant treatment with hydrocortisone. In contrast, the combination of insulin and hydrocortisone induced a significant increase in apo A-I synthesis. Although EGF and insulin increased apo A-IV synthesis, the combination of insulin and hydrocortisone treatment up-regulated apo A-IV synthesis by 2.6-fold. Thyroid hormone treatment lacked effect on the synthesis of any of the apolipoproteins studied. EGF, glucocorticoids, and insulin, along with luminal lipid absorption (27, 28), may play regulatory roles in the developmental expression of apolipoprotein synthesis in the neonatal small intestine. Further studies are underway in this laboratory to delineate the molecular mechanisms of this regulation.

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