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THE EFFECTS OF GASTRIN RELEASING PEPTIDE

IN

FETAL SHEEP

PREGNANT SHEEP

AND

NON PREGNANT SHEEP

by

Pamela Elizabeth Houghton

Department of Physiology

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

August 1990

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ABSTRACT

These studies examined the hypothesis that GRP stimulates insulin release from the endocrine pancreas of ovine fetuses. This could occur due to the action of GRP directly within the fetal compartment or due to indirect effects of GRP on glucose and insulin concentrations in the maternal compartment which in turn influences the availability of glucose for transplacental transfer to the fetus and hence produce alterations in fetal insulin release.

The studies performed provided the following results: (1) IR-GRP was detectable in nerves of the GI tract and in the systemic circulation but not in the pancreas of fetal and adult sheep; (2) Intravenous administration of GRP stimulates insulin and glucagon release and potentiates glucose stimulated insulin release in adult non-pregnant sheep; (3) Preliminary studies suggest that plasma IR-GRP concentrations rise in non-pregnant sheep following feeding. These results are consistent with a proposed incretin action of GRP which acts systemically to alter insulin release. However, further experiments are necessary to confirm this suggestion. The ability of GRP to stimulate insulin release and potentiate glucose stimulated insulin release, is attenuated in pregnant compared to non-pregnant sheep. Furthermore, the diminished insulin response to GRP and/or glucose observed in pregnant sheep was reproduced in non-pregnant ovariectomized sheep treated with progesterone and estradiol but not estradiol alone. This suggests that elevated circulating progesterone concentrations which occur during pregnancy mediate, at least in part, the reduced ability for GRP to influence insulin release observed in pregnant sheep. Similar doses of GRP and/or glucose administered to fetal sheep over the last one-third of gestation did not alter fetal plasma insulin concentrations significantly. IR-GRP is present in very high concentrations in fetal plasma which probably explains why exogenous GRP administration into fetal sheep did not alter circulating GRP or insulin concentrations. Further experiments are necessary to determine the site of production and function of GRP within the fetus.

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LIST OF ABBREVIATIONS

GI .... gastrointestinal tract
GLP .... glucagon like peptide
PP .... pancreatic polypeptide
CCK .... cholecystokinin
VIP .... vasoactive intestinal peptide
PHI .... peptide histidine isoleucine
GRP .... gastrin releasing peptide
CGRP .... calcitonin gene related peptide
VFA .... volatile fatty acids
BLI .... bombesin like immunoreactivity
PL .... placental lactogen
P_4 .... progesterone
E_2 .... estrogen
RNA .... ribose nucleic acid
SEM .... standard error of the mean
RIA .... radioimmunoassay
pO_2 .... partial pressure of oxygen
pCO_2 .... partial pressure of carbon dioxide
IV .... intravenous
MCR .... metabolic clearance rate
GEP .... gastroenteropancreatic peptides
ACTH .... adrenocorticotrophic hormone

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1. HISTORICAL REVIEW

1.1. METABOLISM IN ADULT MONOGASTRIC SPECIES:

1.1.1. OVERVIEW

Despite intermittent feeding circulating blood glucose concentrations must remain within a normal range of concentrations in order to insure that there is a continual supply of glucose to vital organs such as the brain (Unger and Dobbs, 1978; Saudek and Felig, 1976). This task is accomplished within the body by a highly controlled system involving both humoral and neural regulatory mechanisms. Glucoregulatory hormones include insulin, glucagon, adrenaline, and cortisol. Insulin is the dominant hormone which is secreted into the systemic circulation following feeding and acts to lower blood glucose concentrations. The actions of insulin include: (1) decreased hepatic glucose production, via inhibition of glycogenolysis and gluconeogenesis; (2) increased hepatic glucose storage, via stimulation of glycogenesis and formation of glucose derived triglycerides; (3) increased glucose uptake and utilization, in such insulin sensitive tissues as fat and muscle; (4) increased amino acid uptake into muscle cells and stimulation of protein synthesis therein; (5) enhanced glucose and fatty acid uptake and reduced lipolytic processes in adipose tissue (Unger and Dobbs, 1978). Together, these actions of insulin result in the conversion of smaller molecules, such as glucose, amino acids and fatty acids which are absorbed into the blood from the gastrointestinal (GI) tract following feeding, into their respective larger storage molecules of glycogen, protein and triglycerides. This results in a lowering of blood glucose concentrations and nutrient storage.

In the fasted state, substrates are no longer being absorbed from the GI tract and blood glucose levels begin to fall. Glucagon, adrenaline, and cortisol act together in the fasted state to retrieve stored substrates and hence maintain normal blood glucose

concentrations. Glucagon is secreted from the pancreatic A-cell into the portal circulation in response to hypoglycaemia (Walter et al., 1974), acts on the liver via the adenylate cyclase system (Chiasson et al., 1975) to promote hepatic glucose production by stimulating glycogenolytic (Park et al., 1972) and gluconeogenic (Exton, 1969) processes. In addition, glucagon stimulates lipolytic processes by activating the enzyme hormone sensitive lipase present in adipose tissue (McGarry and Foster, 1977). In response to various types of stress, adrenaline and cortisol are secreted from the adrenal medulla and cortex respectively and both act to promote nutrient mobilization and elevate blood glucose concentrations. Adrenaline has similar catabolic effects as glucagon on the liver and on adipose tissue. Cortisol stimulates protein catabolism in muscle cells resulting in the release of free amino acids into the circulation. In the liver, free amino acids are used to produce glucose via hepatic gluconeogenic processes. During times of stress, when cortisol levels are increased, the response of glucagon to stimulation is augmented (Marco et al., 1973; Wise et al., 1973) and the sensitivity to glucagon of its major target organ, the liver, is also enhanced (Park et al., 1972).

To summarize, insulin acts in the fed state to stimulate substrate storage and limits elevations in blood glucose concentrations, whereas glucagon, adrenaline, and cortisol act together in the fasted state to mobilize stored substrates and thereby prevent hypoglycaemia.

1.1.2. PANCREATIC HORMONES

a) Insulin

Insulin is a protein that contains 51 amino acids and consists of two polypeptide chains linked together by two disulphide bonds. The molecular weight of human insulin is 5808 Da. Insulin is synthesized in the pancreatic β-cell in a larger proinsulin precursor

(Tager et al., 1980). During processing and packaging into secretory vesicles, the connecting peptide (C-peptide) is cleaved from proinsulin producing the mature insulin molecule. Upon receiving an appropriate stimulus, the biologically active insulin molecule is then secreted into the circulation along with the C-peptide. The rate of insulin biosynthesis is regulated proportionately by the rate of glucose metabolism within the β-cell (Lin et al., 1972). The nature of this regulation has not been elucidated but when the rate of glucose metabolism in the pancreatic β-cell is stimulated or depressed the rate of insulin synthesis is also stimulated or depressed in parallel (Lin et al., 1972). In addition, cyclic GMP (cGMP) and agents which have been known to stimulate the accumulation of cGMP, inhibit insulin and RNA synthesis in the pancreatic β-cell (Howell and Montague, 1974).

Insulin is released in response to glucose in a biphasic pattern. The first phase is a sharp spike of insulin release which occurs within 2-5 minutes of application of the stimulus. If the stimulus is maintained a second slow gradual rise in insulin secretion occurs (Curry et al., 1986). The mechanism(s) underlying the biphasic insulin response to glucose is equivocal. It is known that the initial phase occurs independent of protein synthesis but the prolonged secondary phase is progressively more dependent on continued protein synthesis (Hedeskov, 1980). One interpretation of two phases of insulin release is that two pools of insulin exist in the β-cell (Grodsky et al., 1969). This hypothesis is supported by ultrastructural studies which reveal that two types of secretory granules (light and dark) are present within the β-cell (Aerts et al., 1980).

The exact mechanism by which glucose stimulates insulin release is not known. The action of glucose on the β-cell is stereospecific which implies that there is a receptor involved (Lin and Haist, 1969). It is also known that in order to elicit insulin secretion, glucose must enter the β-cell via facilitated diffusion (Hellman et al., 1971) and be oxidized

(Grill and Cerasi, 1976). The search for the intermediate glucose metabolite that activates the secretory mechanism has resulted in numerous hypotheses but there is as yet no universal agreement. A glucose-mediated event or combination of events associated with the oxidation of glucose within the β-cell stimulates three intracellular messenger systems: (1) activation of adenylate cyclase (Schuit and Pipeleers, 1985; Grill and Cerasi, 1976), (2) calcium entry into the β-cell (Ashcroft, 1981; Malaisse and Mathias, 1985), and (3) increased phosphatidyl inositol turnover (Hedeskov, 1980). In addition, evidence supports the involvement of calmodulin in insulin release and both tubulin aggregation into microtubules and microfibrillary contraction have been demonstrated to participate in granule movement (Malaisse et al., 1971). Through these intracellular messengers, glucose stimulates both insulin synthesis and secretion (Lin and Haist, 1969).

b) <u>Glucagon</u>

Glucagon is a polypeptide consisting of 29 amino acids (Bromer et al., 1957) with an identical primary structure in all mammalian species thus far studied, with the exception of the guinea pig (Sundby, 1976). Immunoreactive glucagon (IRG) is defined as immunoreactivity measurable with a "C-terminal reacting" antibody which is believed to recognize the C-terminal segment of true glucagon and to react poorly with GLPs (Assan and Slusher, 1972; Heding et al., 1976). It is detectable in totally pancreatectomized patients (Miyata et al., 1976) which suggests that glucagon is produced in additional sites other than the pancreas. Extrapancreatic sites of glucagon production reported so far include the small intestine of the human adult (Kobayashi et al., 1970) and in the gastric fundus of dogs (Larsson et al., 1975). In the dog, glucagon secretion by the stomach does not occur under physiological conditions (Munoz-Barragan et al., 1976) but is a substantial contributor to high circulating levels of IRG in insulin deprived animals whether induced by pancreatectomy (Blazquez et al., 1977;) or by alloxan treatment (Blazquez et al., 1976; Vranic et al., 1974).

Pancreatic glucagon is one of many peptides formed from post translational processing of 179 amino acid pre-proglucagon molecule (Bell et al. 1983). Structural analysis has shown that identical pre-proglucagon mRNAs are present in the pancreas, ileum and colon (Mojsov et al., 1986; Novak et al., 1987). It has been concluded, therefore that the diversity of the molecular forms of the proglucagon-derived peptides in the pancreas and gut arise from different pathways of post-translational processing of the primary transcript. In the A-cell of the pancreas, the prohormone is processed to proglucagon (1-30), which is alos referred to as glicentin-related pancreatic peptide, glucagon, proglucagon (64-69) but the remaining C-terminal fragment proglucagon (72-158) is not further processed (Patzelt and Schiltz, 1984). In the L-cell of the gut, different cleavage sites are used so that proglucagon is processed to proglucagon (1-69; glicentin), proglucagon (33-69; oxyntomodulin), proglucagon (72-108; GLP-1), proglucagon (111-123; an intervening peptide sequence), and proglucagon (126-158; GLP-2). GLP-1 is further cleaved in the gut to GLP-1 (7-37). It is this truncated peptide that is a potent augmentor of insulin release (Conlon, 1988). The factors relating the tissue- specific post-translational processing of proglucagon are not understood but it appears to be tissue specific.

c) Somatostatin

Somatostatin is a tetradecapeptide which was first discovered in the hypothalamus by Brazeau et al., (1973) and subsequently localized by Luft et al. (1974) and Dubois (1975) in cells within the islets of Langerhans. It is present in the pancreatic D-cell or A_1-cell (Orci et al., 1975a). Release of immunoreactive somatostatin into the pancreatic vein is stimulated by perfusion with high concentrations of glucose, arginine, leucine, pancreozymin-cholecystokinin and secretin (Ipp et al., 1977). Further, pharmacological quantities of somatostatin administered by peripheral veins are known to inhibit or reduce

a variety of digestive functions including gastric emptying (Bloom et al., 1975b); the secretion of hydrochloric acid (Bloom et al., 1974), pepsin (Bloom et al., 1975a), and gastrin (Bloom et al., 1974); antral (Boden et al., 1976), duodenal (Boden et al., 1976) and gall bladder motility (Unger and Dobbs, 1978); pancreatic exocrine function (Boden et al., 1976); the secretion of secretin (Boden et al., 1975), motilin (Bloom et al., 1975b), and GLI (Sakurai et al., 1975) by the small intestine; splanchnic blood flow (Wahren, 1976); and absorption of xylose (Wahren, 1976), glucose (Wahren, 1976), and triglycerides (Sakurai et al., 1975). The demonstration that somatostatin inhibits the release of acetylcholine by electrically stimulated parasympathetic nerves (Guillemin, 1976) may, in part, account for many of these inhibitory actions. Together these studies suggest that pancreatic somatostatin acts to restrain the rate of nutrient entry from the GI tract by inhibiting the response of various digestive events to signals from enteric hormones and rising nutrient concentration (Unger and Orci, 1977).

d) Pancreatic Polypeptide (PP)

Pancreatic polypeptide (PP) was isolated initially from chicken pancreas as a by-product of insulin purification by Kimmel and co-workers (1975) and later from pancreatic extracts from several mammalian species including humans (eg. Gersell et al. 1979). PP is a 36 amino acid polypeptide of approximately 4200 Da with no obvious structural similarities between it and other peptides present in the pancreas or GI tract. The C-terminal segment of PP is essential for biological activity and no differences have been reported between several mammalian species including sheep, pig, human, and dogs (Lin et al., 1978). Using immune electron microscopy, PP has been localized within F-cells in the islets of Langerhans (Greider et al., 1978).

The role of PP in regulation of digestion or metabolism has not been established. Intravenous (IV) infusion of PP in dogs at doses that produce serum PP concentrations lower than those found after ingestion of a protein rich meal, produce significant inhibition of basal insulin secretion and insulin secretion that was stimulated by the administration of secretin and cerulin (Taylor et al., 1979). These observations suggest that PP may modulate the pancreatic secretory response to feeding. However, the effects of PP on pancreatic secretions which occur following feeding have not been studied directly.

1.1.3. INTRAPANCREATIC INTERACTIONS

The composition and topographical arrangements of cells within the islets have been studied in a variety of species including humans (Orci, 1976; Orci et al., 1975b & c, 1976a). In rodents, insulin containing β-cells form the central mass and constitute approximately 60 % of the islet cells. The most peripherally situated cells are glucagon-containing A-cells which form a ring one or three cells in thickness, and comprise approximately 25 % of the endocrine cells. In islets located in the paraduodenal or head portion of the pancreas, the peripheral glucagon containing A-cells appear to be replaced by F-cells containing PP. Between the outer mantle of A-cells are the somatostatin containing D-cells. Although there are species differences with respect to the relative distribution within the islets, in all species studied to date, β- A- D- and PP- cells are present and β-cells predominate.

Results from earlier studies suggest that there is considerable interaction between cells of the islets of Langerhans. Small changes in the concentrations of intrapancreatic hormones, such as glucagon and somatostatin, influence β-cell secretion in a paracrine manner. Glucagon released from the pancreatic A-cell is known to directly stimulate insulin release (Samols et al., I965) and small changes in intraislet insulin concentration

are known to suppress glucagon secretion (Samols et al., 1965). Somatostatin release from the D-cell can be stimulated by modest increases in local glucagon levels (Kawai et al., 1982) and can act to inhibit both glucagon and insulin release (Kawai et al., 1982; Samols et al., 1976). A recent study by Samols and co-workers demonstrated that anterograde infusion of glucagon antibody into rat pancreatic islets did not affect insulin secretion but rapidly decreased somatostatin secretion, whereas, retrograde infusion of glucagon antibody decreased insulin secretion but had no effect upon somatostatin secretion (Stagner, Samols, and Marks, 1989). These results confirm that islet capillary blood flow occurs from the β-cell core to the A- and D-cell mantle and cast doubt on the physiological significance of the results obtained from earlier studies. Rather these studies suggest that, within the islet, vascular borne insulin regulates the release of glucagon, which in turn, regulates the release of somatostatin. Furthermore, somatostatin appears to be vascularly neutral owing to its downstream position in the sequence (β to A to D) of cellular position.

Intracellular routes of communication within the pancreatic islets include tight junctions (Orci et al., 1973a) which are linear points of fusion between the outer leaflets of the plasma membranes of two adjacent cells, and gap junctions (Orci et al., 1975c and 1973c) which are low resistance pathways through which molecules of less than 1000 Da can move from the cytosol of one cell to that of an adjacent cell without entering the intercellular space. In addition, islet cells share desmosomes which are primarily involved in cell to cell adhesion. Although the precise function of tight and gap junctions in the islets of Langerhans remains to be determined, the fact that they are present in the islets of all species studied to data suggests that they have special functional significance. It has been suggested that the tight junctions can act as barriers for diffusion which may constitute a vital method for compartmentalizing secretion products of the islet cells that

would otherwise have profound influences on neighbouring islet cells (Orci and Unger, 1975). In addition, a high degree of coordination between islet cells rendered possible by gap junctions and by paracrine and endocrine actions of islet cell hormones upon the islet cells, could represent an important means of achieving the appropriate mixture of insulin, glucagon, and somatostatin that is secreted. This suggestion is supported by reports of persistent hyperinsulinaemia and hypoglycaemia in patients with Nesidiobastosis syndrome which have disorganized islets due to improper development of the endocrine pancreas (Hirsh et al., 1977; Kloppel et al., 1974). This observation suggests that proper intrapancreatic organization of the endocrine cells must exist to permit appropriate paracrine interactions and that these interactions are necessary for the endocrine pancreas to function optimally in the maintenance of nutrient homeostasis.

1.1.4. METABOLIC CONTROL OF PANCREATIC ISLET FUNCTION

The enteroinsular axis as named by Unger and Eisentraut (1969) includes all stimuli from the gut to different cell types of the pancreatic islets. The stimuli arising from the GI tract can be divided into three general types: (1) substrate stimulation; (2) hormonal stimulation; and (3) neural stimulation.

It has long been established that substrates, such amino acids, fats, and glucose, are absorbed from the GI tract and circulate to the pancreas where they are capable of influencing pancreatic endocrine secretions. Both glucose and many different amino acids, particularly arginine, stimulate insulin release. Amino acids including lysine, arginine, phenylalanine, and tryptophan, also augment glucose-stimulated insulin release (Floyd et al., 1966). Studies performed in man and in dog have reported that changes in circulating fatty acids did not alter insulin secretion in those species (Brown et al., l975). Others have shown that IV administration of fat into dogs (Coren et al., 1972) and baboons (Madison

et al., 1968) elevated serum insulin levels. Most investigators believe that fats are not important physiological regulators of insulin release in monogastric animals. Glucose is thought to be the most important metabolic regulator of insulin release. It potently stimulates insulin release from β-cells and inhibits glucagon release from A-cells. In addition, glucose modulates the responsiveness of the β-cell to other neural and humoral agents (H ɔdeskov, 1980).

1.1.5. ENDOCRINE CONTROL OF PANCREATIC ISLET FUNCTION

Carbohydrates, amino acids and fats, present in the lumen of the GI tract following feeding can alter the activity of local endocrine and/or neural cells which in turn can transmit information to the pancreas and influence pancreatic endocrine secretion (Creutzfeldt, 1979). The presence of these interactions was first suggested in 1877 by Claude Bernard in his "Lecons sur la diabete" in which he reported the observation that much larger amounts of glucose could be given orally than intravenously without the production of glucosuria (cf. Bernard 1979 & 1980). He explained these differences by the fact that glucose was largely metabolized during the first passage through the liver and thus hyperglycaemia was prevented.

Bayliss and Starling (1902) demonstrated that introduction of hydrochloric acid into a denervated intestinal loop stimulated exocrine pancreatic secretion similar to that seen after intraduodenal acidification. Since all nervous connections were considered to have been severed in this preparation, Bayliss and Starling concluded that an active agent was carried by the bloodstream from the jejunum to the pancreas. This active agent was later found to be secretin. Bayliss' and Starling's demonstration that secretin acts via humoral mechanisms was a seminal event and certain investigators mark this discovery as the beginning of the field of endocrinology. Starling referred to these blood borne messengers

as "hormones". The word hormone, coined by W. B. Hardy, was taken from the greek word usually translated as "I arouse to activity". In the 1915 edition of his textbook, Bayliss characterized hormones as substances that were blood borne messengers (Bayliss, 1915). Investigators have since regarded the word hormone as implying a blood borne mode of delivery for chemical messengers. It has recently been recognized that biologically active peptides present in the GI tract and elsewhere may exert regulatory functions by non-humoral as well as humoral mechanisms. As a result, this definition for the word hormone has been expanded by some investigators to include substances which act as a chemical messengers regardless of their mode of delivery to target organs (Mutt, 1983). The following subgroups have also been defined. Endocrine substances (hormones) exert their effects over long distances via the bloodstream, paracrine substances are secreted by one cell and exert their biological effects on neighbouring cells by local diffusion, and autocrine substances act back on the cells that secrete them. In addition, chemical messengers are used to transmit information between nerve cells in which case they are referred to neuromodulator or neurotransmitter substances.

As early as 1906, Moore hypothesized that a chemical regulator of pancreatic secretions was present in the duodenum and was responsible for an association between the GI tract and pancreas which diabetics lacked and thus had glucosuria (Moore et al., 1906). To test this hypothesis Moore and his co-workers administered duodenal extracts to diabetics but only inconclusive results were obtained (Moore et al., 1906). Interest was not revived until twenty years later when insulin was discovered in the endocrine pancreas and shown to have potent glucose lowering abilities (Banting and Best, 1922). LaBarre and his colleagues (1930) performed cross circulation experiments and demonstrated that the IV injection of crude secretin extract contained two active principles: one that stimulated pancreatic exocrine secretions which they called "excretin" (also known as secretin) and

another that stimulated pancreatic endocrine secretions which LaBarre termed "incretin". Similarly, Heller in 1929 also prepared a duodenal extract which was capable of lowering blood glucose concentrations and called it "duodenin" (cf. Creutzfeldt, 1979). For the next thirty years "incretin" and "duodenin" were forgotten. Interest was revived following the development of radioimmunoassay (RIA) techniques which allowed measurement of plasma insulin concentrations. McIntyre and colleagues (1964), Erlick and colleagues (1964), and Dupré and co-workers (1964a) demonstrated that a much greater insulin response was observed after an oral glucose load than following an IV injection of the same amount of glucose, despite a smaller increase in blood glucose levels. In addition, Dupré and co-workers demonstrated that the infusion of glucose directly into the duodenum, but not liver, was found to produce a similar augmented elevation in plasma insulin concentrations as those evoked following oral ingestion of glucose (Dupré, 1964b) and that an extract of intestinal mucosa stimulated insulin release (Dupré and Beck, 1966). Taken together these experiments suggest that the direct effects of nutrients and their metabolites on the pancreas cannot account adequately for the regulation of insulin secretion. Further, these differences cannot be due simply to first pass hepatic uptake of glucose entering the portal circulation but rather the small intestine is the source of some factor, named "incretin", which is responsible for enhanced pancreatic endocrine secretion. This result stimulated a massive search to identify the incretin. The following criteria were established for incretin candidates by Creutzfeldt (I976). Incretin is (1) an endocrine transmitter produced in the GI tract, (2) it is released by oral or intragastric but not IV administration of nutrients, especially carbohydrates, and (3) it is capable of augmenting glucose-stimulated insulin release when exogenously infused in amounts not exceeding physiological concentrations. Recently, several incretin candidates have been identified in the GI tract. Collectively these factors are referred to as GI peptides (Brown

and Otte, 1978). Despite the fact that the presence of an incretin was suggested almost a century ago, the identity of this humoral agent has yet to be determined. This is probably a reflection of the technical problems which exist in studying GI peptides. These include: the fact that GI peptides (1) are not present in discrete glands but rather are spread diffusely throughout a number of tissues in the body, not only in endocrine-type cells but also in neural structures, (2) are present in relatively low tissue concentrations in an organ well suited for proteolytic degradation, (3) have overlapping bioactivities as a result of structural homology with one another, (4) may exert their regulatory functions by various transport mechanisms; in certain cases they may be delivered to target organs via the bloodstream (ie. humoral mechanisms) but in other instances by strictly local release mechanisms (ie., paracrine, neuromodulatory / neurotransmission mechanisms). As a result of these complications, experiments employing glandular extirpation and exogenous hormonal replacement therapy, which were eminently successful in establishing the physiology of hormones present in discrete endocrine glands, were, for obvious reasons, unavailable for the study of the GI hormones.

Historically gastric inhibitory peptide (GIP) has been thought to be the most likely to play an important physiological role in the augmentation of glucose-stimulated insulin release. GIP was first identified in the GI tract by Dupré (l964a) and subsequently was isolated and characterized by Brown, Dupré and others in 1975. It has been shown to stimulate insulin release in a variety of species (Rabinovitch and Dupré, l974) and both *in vitro* (Cataland et al., l974) and *in vivo* (Pederson et al., 1975) experiments have clearly demonstrated that the insulinotropic effect of GIP is glucose dependent. Hence, the peptide has been renamed glucose dependent insulinotrophic peptide (Pederson et al., 1975). In addition, plasma concentrations of GIP are elevated following oral (Kuzio et al., l974) but not IV (Ebert and Creutzfeldt, l980 and 1982) glucose administration. Therefore,

GIP has fulfilled all the criteria as an incretin as defined by Creutzfeldt (Creutzfeldt, 1976). However, GIP cannot be the sole incretin, since the administration of GIP antiserum reduced, but did not abolish, the incretin effect seen following intraduodenal glucose administration (Ebert and Creutzfeldt, 1982) and did not alter the oral glucose tolerance test (Ebert and Creutzfeldt, 1983). More recently GLP-1(7-37) has been implicated as a strong incretin candidate (Kreymann et al. 1987). Other suggested incretin candidates include secretin, gastrin, cholecystokinin (CCK), PP, and enteroglucagon (McDonald, 1986; Dupré, 1980). However, these GI peptides either stimulated insulin release only at supraphysiological concentrations or they did not augment glucose-stimulated insulin release. The possibility of several incretin candidates acting synergistically under physiological conditions to produce the total incretin effect cannot be ruled out.

1.1.6. NEURONAL CONTROL OF PANCREATIC ISLET FUNCTION

a) The Autonomic Nervous System

Although several factors responsible for the endocrine transmission between the GI tract and pancreas have been proposed and investigated, there is little information available about neural transmission. In 1849, Claude Bernard demonstrated that dogs became diabetic following the puncture of the floor of the fourth cerebral ventricle. This information provided the first evidence of involvement of the nervous system in the control of metabolic processes (cf. Creutzfeldt, 1979).

Whether the nervous innervation of the endocrine pancreas plays an important physiological role in the control of metabolic processes is controversial. Glucose tolerance and insulin response to oral glucose but not IV glucose have (Jakob et al., 1970) and have not (Creutzfeldt and Ebert, 1985; Lindkaer-Jensen et al., 1976) been shown to be reduced after pancreatectomy and orthotropic transplantation of a denervated pancreatic

graft. Conflicting results may be due partially to organ damage and/or concomitant immunosuppressive treatment.

Stimulation of the sympathetic splanchnic nerve or mixed pancreatic nerve in atropinized animals results in decreased insulin release and increased glucagon release (Marliss et al., 1973). This effect is also seen when catecholamines are administered either *in vivo* or *in vitro* (Woods and Porte, 1974). Vagal stimulation (Woods and Porte, 1974) and administration of parasympathomimetic drugs (Woods and Porte, 1974) stimulates insulin release in a glucose dependent manner (Malaisse et al., 1976). Further, neurotransmitter blocking experiments suggest that in several monogastric species, α-adrenergic stimulation decreases insulin release (Porte and Robertson, 1973), β-adrenergic stimulation increases insulin release (Robertson and Porte, 1973), and acetylcholine acting on muscarinic receptors increases insulin release (Frohman et al, 1967). The results from these experiments involving sectioning and stimulation of nerve trunks innervating the pancreas as well as experiments involving the administration of cholinergic and adrenergic drugs and neurotransmitter blocking agents, collectively provide evidence to support the suggestion that the autonomic nervous system does play an important role in the control of endocrine pancreatic function.

At present, it is unknown whether stimulating cholinergic and adrenergic nerves produce a direct effect on the pancreatic islets or whether they only activate intrinsic pancreatic peptidergic nerves which in turn influence insulin output after ingestion of nutrients. Both adrenergic sympathetic nerves and cholinergic parasympathetic nerves directly innervate the pancreatic islets by way of the splanchnic and vagus nerves respectively (Coupland, 1958). These nerve terminals end blindly underneath the basal lamina surrounding the islet (Fujita et al., 1976) cells, suggesting that on release, neurotransmitters may diffuse through the extracelluar space and exert their effects directly on a large number of islet cells simultaneously.

Vagally mediated insulin release from the pancreas, or in response to the presence of food in the oropharynx and esophagus or to the sight, smell, or thought of food is termed "cephalic insulin phase". The cephalic phase of insulin release has been well documented in several species including dogs (Hommel et al., 1972), rats (Louis-Sylvestre, 1978), and man (Parra-Covarrubias, 1971). However, the relative contribution of cephalic insulin secretion is controversial (Sjostrom et al., 1980; Taylor and Feldman, 1982).

Stimulation of parasympathetic or sympathetic neural innervation to the GI tract may also participate in GI enhancement of insulin release by controlling the release of certain insulinotropic GI peptides such as GIP. GIP response to intraduodenal glucose perfusion was shown to decrease after atropine administration (Henderson et al, 1976) which suggests that GIP release is regulated by nerves.

b) <u>Peptidergic Nervous System</u>

In addition to established cholinergic and adrenergic nerves, peptidergic nerves have been identified within intrinsic neural structures of the gut and pancreas. GI peptides known to be present in pancreatic nerves and capable of stimulating pancreatic endocrine secretions include: CCK octapeptide (CCK-8; Larsson and Rehfeld, 1979), enkephalins (Hart and Cowie, 1978), vasoactive intestinal peptide (VIP; Schebalin et al., 1977), peptide histidine isoleucine (PHI; Szecowka et al., 1983), and gastrin releasing peptide (GRP; McDonald et al., 1981 and 1983). Neuropeptides present in the gut and pancreas which have been shown to inhibit insulin release include: neurotensin (Brown and Vale, 1976), somatostatin (Hokfeldt et al., 1975), substance P (Brown and Vale, 1976), calcitonin gene related peptide (CGRP; Hermansen and Ahren, 1990) and galanin (McDonald et al., 1985). There is little information on the role of these peptides in the neural transmission between the gut and the pancreas. This may be partially due to the fact that changes of

plasma levels cannot be used to assess the physiological role of peptide neurotransmitter suspected of incretin activity. Also it may well be that a peptide present in the nerves of gut and pancreatic islets such as GRP, which has *in vivo* and *in vitro* insulin releasing potency, is an important component of the enteroinsular axis without showing alterations of its plasma levels. The peptidergic neural system may participate in the regulation of insulin secretion in several ways: (1) by directly regulating the secretion of GI insulinotropic peptides into the blood; (2) by directly regulating the secretions of the pancreatic islet cells by local release from nerve terminals; (3) by functioning as neurotransmitter/neuromodulators in interneurons and indirectly regulating islet cell function via mechanisms described in 1 & 2. (4) by acting as a neurohormone which is secreted into, and circulates within, the systemic circulation and acts in an endocrine fashion to alter pancreatic endocrine secretion.

1.1.7. GASTRIN RELEASING PEPTIDE (GRP)

GRP is a 27 amino acid peptide first isolated from porcine non-antral tissue (McDonald et al., 1979) and due to structural and functional similarities is believed to be the mammalian analogue of the amphibian molecule - bombesin (McDonald et al., 1981 & 1983). The C-terminal region of GRP which is essential for bioactivity, is highly conserved between species and between GRP and other members of the bombesin family of peptides (McDonald et al., 1979). This phylogenetically conserved peptide has been demonstrated in nerves in the hypothalamus and pituitary (Brown et al., 1978), female urogenital tract (Ghatei et al., 1985), GI tract (Yanaihara et a., 1981; Buffa et al., 1982) and pancreas (Moghimzadeh et al., 1983; Holst et al., 1983; Ghatei et al., 1984). In addition, GRP has been found within endocrine-like cells in human pulmonary tissue (Polak et al., 1978). Although it has been reported that GRP is present in endocrine cells

of the GI tract (Polak et al., 1978; McCrossan et al., 1977), most investigators believe that GRP is associated exclusively with neural structures in the GI tract and pancreas (Walsh et al., 1979; McDonald et al., 1981). GRP has been localized using immunocytochemical techniques in the intra-pancreatic ganglia and in nerve fibers penetrating the pancreatic islets of the pig (Ghatei et al., 1984; Greeley and Thompson, 1984) and dog (McDonald and Buchan, unpublished results). IV adminstration of GRP produces a wide spectrum of effects. GRP stimulates gastric and pancreatic exocrine secretions (cf. McDonald, 1988) and causes the release of several peptides from the GI tract and pancreas including; CCK (cf. McDonald, 1988), gastrin (McDonald et al., 1981; Varner et al., 1981), PP (McDonald et al., 1983; Kaneto et al., 1978), motilin (Knigge et al., 1984), glucagon (McDonald et al., 1981 and 1983; Kaneto et al., 1978, Knigge et al., 1984), neurotensin (Knigge et al., 1984), GIP (McDonald et al., 1981, 1983; Greeley et al., 1986; Becker et al., 1978), in humans and in several experimental animals including dogs (McDonald et al., 1981; Kaneto et al., 1978), mice (Pettersson and Ahren, 1987), and pigs (Knuhtsen et al.,, 1987). Further, the insulinotropic effect of GRP is glucose dependent in rats (Greeley and Thompson, 1984; Martindale et al., 1984) and in man (Wood et al., 1983). GRP stimulates insulin release following application to isolated perfused pancreas (Ipp and Unger, 1979; Martindale et al., 1982), isolated islets (Alwmark et al., 1986), and a neoplastic β-cell line (Swope and Schonbrunn, 1984). In addition, parasympathetic nerve stimulation of isolated perfused porcine pancreas causes atropine resistant elevations in GRP and insulin concentrations in the pancreatic venous effluent (Knuhtsen et al., 1984).

There is little information about the mechanism of action of GRP but it appears, in these monogastric species, that this mechanism probably involves specific delivery of GRP to target organs via local release from non-cholinergic extrinsic neural structures rather than by release into the systemic circulation. Consistent with a local release

mechanism, no convincing evidence for elevation of GRP levels in plasma following a meal was found in the human (Varner et al., 1981), dog (McDonald et al., 1983), or pig (McDonald, personal communication). However the possibility that this may occur in other species cannot be ruled out.

These studies clearly show that GRP when infused intravenously, is a potent insulinotropic agent in several different monogastric species. Whether or not GRP plays a physiological role in the regulation of the endocrine pancreas remains to be determined. Studies to address this question await the acquisition of a potent and specific GRP antagonist.

1.2. RUMINANT METABOLISM

1.2.1. OVERVIEW

In ruminants, most dietary carbohydrate is fermented to volatile fatty acids (VFA) which include the short chain fatty acids acetate, proprionate, and butyrate (Annison and Armstrong, 1970) by microbial activity in the rumen. As a result, ruminants absorb little glucose from their GI tract (Bergman, 1973). Since their glucose requirements are similar to that in non-ruminant animals (Ballard et al., 1969), glucose needs in these animals must be met via gluconeogenic processes. In ruminants, hepatic glucose output (primarily gluconeogenesis) is greatest following feeding (Bergman et al., 1970), whereas in non-ruminants gluconeogenesis is greatest during starvation (Shoemaker, et al., 1963). In ruminants, the major gluconeogenic substrates are proprionate, lactate/pyruvate, amino acids, and glycerol (Bergman, 1973). Proprionate and amino acids absorbed from the GI tract are the major glucose precursors in the fed animal whereas during starvation the major precursors must be supplied by peripheral stores. Since lipid stores provide only a small amount of substrates as glycerol (Bergman et al., 1968), amino acids and hence peripheral protein must be the major substrates for gluconeogenesis during starvation.

Acetate and especially butyrate, two end products of carbohydrate fermentation, can be converted to ketone bodies (acetoacetate and β-hydroxy butyrate), during absorption across the rumen epithelium (Brockman, 1976). In fact, alimentary ketone body release has been reported to account for 7% (Baird et al., 1975) and as much as 30% (Pethick et al., 1983) of ruminant energy requirements. Circulating ketone bodies are 4-5 fold greater in adult sheep than concentrations present in non-ruminants (Brockman, 1976). These ketone bodies can be used as oxidizable substrates in heart (Williamson and Krebs, 1961), kidney (Weidemann and Krebs, 1969), skeletal muscle (Soling and Kleineke, 1972), and the lactating mammary gland (Hawkins and Williamson, 1972). In this way, ketone bodies can act to spare the use of glucose for tissues that require it such as the brain. The rate of utilization of ketone bodies by tissues is entirely dependent on circulating ketone body concentration (Kaufman and Bergman, 1971). Since plasma concentrations of fats and ketone bodies are elevated during pregnancy (Heitmann et al., 1987), ketone bodies become an increasingly important energy substrate in ruminants throughout gestation (Pethick et al., 1983).

1.2.2. ACTIONS OF PANCREATIC HORMONES

As in monogastric animals, there is a concomitant elevation in plasma insulin and glucagon concentrations in ruminants following feeding. However, in ruminants peak insulin and glucagon concentrations do not occur until 2 - 4 hours after the meal (Brockman, 1978) whereas in simple stomached animals they occur relatively soon following eating. Also in contrast to monogastric animals, changes in plasma insulin concentrations which occur following feeding of ruminants, correlate poorly with changes in blood glucose and amino acids, but are closely associated with changes in plasma VFA concentrations (Brockman, 1978).

a) **Actions of Insulin**

In ruminants, insulin inhibits hepatic glucose production by only a small extent (Brockman et al., 1975b; West and Passley, 1967) and the action of insulin on peripheral tissues, such as fat and muscle, in promoting glucose uptake and utilization, is slower than in non-ruminants (Jarret et al. 1974). These data suggest that in ruminants insulin has a greater effect on the disposal of glucose at non-hepatic sites than on hepatic output of glucose. The effect of insulin on lipid metabolism in ruminants is less clear. It is known that insulin infusion into alloxan-diabetic sheep increases acetate uptake by peripheral tissue (Jarret et al., 1974), and decreases plasma acetate concentrations. Also, insulin administration into sheep reduces plasma concentrations of glycerol (Bauman, 1976) and free fatty acids (West and Passley, 1967) and decreases net output of glycerol (Brockman et al., 1975a) and free fatty acids (Khachadurian et al., 1966) from adipose tissue. These *in vivo* studies suggest a role for insulin in promoting net lipogenesis in non-hepatic tissues of sheep. However, *in vitro* studies have not confirmed a lipogenic role for insulin (Khachadurian et al., 1967).

Insulin also influences protein metabolism in ruminants. It increases plasma amino acid concentrations (Brockman et al., 1975a; Call et al., 1972), amino acid uptake into muscle but not liver (Brockman et al., 1975a), and enhances the incorporation of amino acids into muscle protein (Jefferson et al., 1974).

Overall, insulin appears to direct its influence on metabolism primarily at non-hepatic sites. It directs the movement of amino acids, glucose, and fats into muscle and adipose tissue. In its absence synthetic activity is reduced in these peripheral tissues and there is a net movement of metabolites from muscle and adipose tissue to the liver.

b) Actions of Glucagon

Glucagon increases blood glucose levels in ruminants by acting primarily on the liver where it promotes glucose output via the stimulation of glycogenolysis and gluconeogenesis (Brockman and Bergman, 1975). Evidence suggests that hepatic effects of glucagon are mediated by the adenylate cyclase system (Atwal and Sauer, 1973) and are associated with increased activity of pyruvate carboxylase (Brockman and Manns, 1974), a key gluconeogenic enzyme.

Glucagon infusion *in vivo* results in increased plasma fatty acid (Bassett, 1971) and glycerol (Brockman, 1978) concentrations, provided glucagon-stimulated insulin secretion is prevented. *In vitro* studies however have not confirmed the lipolytic role for glucagon in ruminants (Bauman, 1976). Thus, the action of glucagon on lipid metabolism is unclear but it is apparent that if glucagon has an effect on adipose tissue in ruminants, then it is not as potent as insulin. Glucagon administration to sheep promotes the uptake of glucogenic amino acids by the liver (Brockman et al., 1975a) and enhances the conversion of alanine to glucose (Brockman and Bergman, 1975) which in turn reduces plasma amino acid concentrations. Thus, in ruminants, glucagon exerts its effects predominantly on the liver while insulin seems to act primarily at non-hepatic sites.

1.2.3. FACTORS AFFECTING INSULIN AND GLUCAGON RELEASE

a) Substrates

Factors responsible for the stimulation of pancreatic endocrine secretions are similar in ruminant and non-ruminants species. Elevations in plasma concentrations of glucose and various amino acids including arginine, leucine, and phenyl-alanine, stimulate insulin release in adult non-pregnant sheep (Davis et al., 1972). Differences between ruminants and non-ruminants lie in the fact that free fatty acids seem to play an important

physiological role in the regulation of insulin, and possibly glucagon secretion, in ruminants (Brockman, 1978) but do not affect pancreatic endocrine secretions in non-ruminants (Brown et al., 1975). Brockman (1978) has shown that administration via a mesenteric catheter of proprionate and butyrate to sheep at physiological entry rates is associated with significant elevations in insulin and glucagon concentrations. In addition, plasma VFA concentrations following feeding are elevated significantly with insulin and glucagon concentrations (Brockman, 1978).

b) <u>Nerves</u>

The autonomic nervous system has also been implicated in the control of endocrine pancreatic secretions in ruminants. Electrical stimulation of the splanchnic nerves of calves (Bloom et al., 1973) as well as adrenaline infusion into sheep (Bassett, 1971) and calves (Bloom et al., 1973), stimulates glucagon secretion and inhibits basal insulin release. Fowden (1980b) has also found that adrenaline administration inhibits glucose-stimulated insulin release. In addition, stress caused by anaesthesia and hypoxia which are known to stimulate sympathetic nerve activity and elevate circulating catecholamine concentrations (Jones et al., 1988), also stimulate glucagon and inhibit insulin secretions in sheep (Brockman and Manns, 1976) and calves (Bloom et al., 1975).

Stimulation of the vagus nerve in conscious calves results in elevations in plasma insulin, glucagon, PP, and glucose concentrations (Adrian et al., 1983). Atropine administration reduces basal glucagon concentrations in intact and sympatectomized calves, impairs glucagon response to hypoglycaemia in sympatectomized calves (Bloom et al., 1974), and blocks the insulin release following feeding in sheep (Bassett, 1974). These experiments suggest that the parasympathetic nervous system may be involved in the regulation of circulating glucagon concentrations and responsiveness of the A-cell to other glucagon secretagogues in calves and in the insulin response to feeding in sheep.

c) <u>GI Peptides</u>

Although GI peptides are believed to play an important role in the control of pancreatic endocrine secretions in non-ruminants, few studies have examined their effect in ruminants. Immunocytochemical studies have revealed that many of the GI peptides known to be present in monogastric species are distributed in corresponding locations in the GI tract of the adult sheep (Calingasan et al., 1984). Electrical stimulation of the vagus nerve of conscious calves not only induces pancreatic endocrine secretions, but also stimulates the release of gastrin, CCK, somatostatin, and VIP from the GI tract but did not alter plasma concentrations of GIP or GRP (Adrian et al., 1983). Bladin et al (1983) found that plasma gastrin and PP concentrations were also elevated in response to vagal stimulation in sheep. Splanchnic nerve stimulation caused elevations in plasma GRP and neuropeptide Y (NPY) concentrations but no change in VIP or CGRP concentrations in conscious calves (Bloom et al., 1988). Thus, GI peptides are known to be present within the GI tract of ruminants and can be released upon nervous stimulation, but studies examining the effects of these peptides on insulin release are sparse.

The role of GIP in the ruminant is unknown. In contrast to the simple stomached animals, the secretion of GIP is not stimulated following intraduodenal administration of glucose or fatty acids in either young and adult goats or in calves (Nilssen et al., 1983) nor are plasma concentrations of GIP altered following feeding of adult sheep (Bunnet and Harrison, 1986). Furthermore, Nilssen and co-workers (1983) have reported that GIP does not appear to stimulate the secretion of insulin in ruminant animals. Thus, although GIP is important in augmenting the insulin response to an oral glucose challenge in non-ruminants, it does not appear to play such a role in ruminants.

It is known that plasma gastrin concentrations are elevated after eating in sheep at a time when insulin and glucagon concentrations also are increased (Bloom et al.,

1975a). Since plasma gastrin concentrations are also elevated following sham feeding and by vagal stimulation, Bloom and co workers have suggested that gastrin plays a physiological role in mediating the cephalic phase of insulin release. This seems unlikely however since supraphysiological concentrations of gastrin are required to stimulate insulin release. Bloom and co-workers have also reported that neurotensin known tc be present in the ileum, is released into the circulation following feeding, and is capable of stimulating glucose dependent insulin release in calves (Bloom et al., 1984). From these experiments it seems possible that neurotensin, and possibly gastrin, may play a physiological role in the control of insulin release in ruminants. However the physiological significance of these responses remains to be determined. Studies examining the physiological role of other GI peptides in ruminants are also required.

d) Gastrin Releasing Peptide (GRP)

In common with monogastric animals, IV GRP administration to conscious calves produced dose dependent elevations in plasma insulin concentrations (Bloom et al., 1983; 1979 a & b) and GRP-stimulated insulin release is dependent on circulating concentrations of glucose and amino acids (Bloom et al., 1984). Bloom and co-workers have performed several studies examining the role of GRP in the control of endocrine pancreatic secretion in conscious calves (Bloom et al, 1983 a,b and 1984). From these studies it appears that in this ruminant species GRP is present in the systemic circulation and therefore may act in an endocrine fashion to exert its insulinotropic effect on the pancreas. This suggestion is supported by immunocytochemical studies which have demonstrated the presence of GRP in the GI tract but not pancreas of the ruminant (Bloom et al., 1983a & b; Dockray et al., 1979). Further differences between ruminant and non-ruminant animals lie in the fact that, in calves, elevations in circulating GRP concentrations were produced by splanchnic nerve stimulation (Bloom et al., 1980), but not by vagal stimulation (Adrian et

al., 1983) which is reported to increase GRP concentrations in monogastric animals (Knuhtsen et al., 1985).

Bloom and his colleagues (1984) have also suggested based on preliminary studies that feeding of calves which is associated with increased sympathetic discharge, produced significant increases in circulating plasma bombesin-like immunoreactivity (BLI). If completed studies confirm these preliminary reports then based on Creutzfeldt's criteria (Creutzfeldt, 1976), GRP is a strong incretin candidate in conscious calves. This is an interesting hypothesis since GIP which is considered to be the most probable incretin in monogastric species (Brown and Otte, 1978), appears not to play a role in the control of insulin release in ruminants (Nilssen et al., 1983; Bunnett and Harrison, 1986). Perhaps GRP predominates as the major incretin in this species. Whether GRP acts similarly in other ruminants, such as the sheep, remains to be determined.

1.3. METABOLISM DURING PREGNANCY

1.3.1. OVERVIEW

Pregnancy is associated with marked changes in metabolism. There are alterations in the deposition of every major class of nutrient during late gestation. These changes in metabolism which occur during pregnancy involve alterations which are biphasic. The first half of pregnancy is characterized by β-cell hyperplasia (Van Assche et al., 1980), increased insulin secretion in response to glucose (Buch et al., 1986), and as a result progressive hyperinsulinaemia (Spellacy and Goetz, 1963). At the same time, there are increases in tissue glycogen storage (Paul, 1972) and peripheral glucose utilization (Silverstone et al., 1961) and decreases in hepatic glucose production and maternal fasting plasma glucose concentrations (Silverstone et al., 1961; Bleicher et al., 1964). There is a minimal increase in total peripheral insulin resistance during this first

stage of pregnancy (Flint et al., 1979). In addition, there is normal or slightly improved carbohydrate tolerance (Baird, 1986) and normal or increased removal of triglycerides from the circulation (Knopp et al., 1973). The net effect is increased storage of energy in the form of fat.

As pregnancy progresses, there is a shift toward increased production of free fatty acids and ketones (Knopp et al., 1973, Kuhl and Holst, 1976). Pancreatic islet cell hypertrophy continues and insulin responses to glucose increase (Baird, 1986). There is relative fasting hypoglycaemia (Baird, 1986), elevations in plasma lipids (Bleicher et al., 19645), hypoaminoacidaemia (Kalkhoff et al., 1964), and marked sensitivity to food deprivation (Freinkel, 1980).

In late pregnancy when the fetus is growing rapidly, maternal plasma insulin concentrations remain raised (Bleicher et al., 1964, Spellacy and Goetz, 1963, Kalkhoff et al., 1964) but the number of insulin receptors on adipocytes falls to non-pregnant levels (Knopp et al., 1973) and peripheral insulin resistance develops (Burt, 1956; Bleicher et al., 1964). In addition, plasma glucagon concentrations are slightly but significantly increased during fasting (Kuhl and Holst, 1976), are suppressed to a greater extent after oral glucose administration (Kuhl and Holst, 1976), and have (Kitzmiller et al., 1980) and have not (Hornnes et al., 1981) been shown to be elevated to significantly greater levels following oral administration of amino acids. These changes in the action of the glucoregulatory hormones during late gestation lead to (1) reduced assimilation of glucose and triglycerides by the maternal tissues (2) increased transfer of glucose to the fetus, (3) increased lipolysis (4) decreased stores of hepatic glycogen and (5) increased production of hepatic glucose. Maternal metabolism therefore copes with the increased outpouring of glucose to the fetus during the second half of pregnancy, by switching to the use of fat to fuel the metabolic processes. Such changes collectively result in the development of

a diabetic-like state during pregnancy which is characterized by an impaired oral glucose tolerance test.

Much less is known about protein metabolism during pregnancy. Studies performed in rats suggests that changes in protein metabolism which occur during pregnancy are also biphasic in nature. In the early phase of pregnancy protein storage mechanisms predominate whereas in late pregnancy protein breakdown occurs (Naismith and Morgan, 1976).

1.3.2. FACTORS RESPONSIBLE FOR METABOLIC CHANGES DURING PREGNANCY

The mechanisms underlying these metabolic changes which occur during pregnancy are still obscure. Possible explanations include: (1) increased secretion of proinsulin or increased rate of insulin degradation; (2) peripheral insulin resistance; and (3) increased secretion of hormones with an anti-insulin effect particularly Placental Lactogen (PL), estrogen (E_2), progesterone (P_4), glucagon, and cortisol); or (4) a combination of two or more of these mechanisms.

1.3.2.1. Increased Secretion of Proinsulin or Rate of Insulin Degradation

Kuhl et al (1976) have demonstrated that the proportion of less active proinsulin molecules in the plasma does not change during pregnancy and despite reports of increased insulin degrading activity in both fetal and placental tissue (Freinkel and Goodyer, 1980), most reports in both animals and human pregnancy are unanimous that the overall metabolic clearance rate, distribution space, and biological half-life of insulin are not significantly altered by normal pregnancy (Bellman and Hartmann, 1975; Burt and Davidson, 1974). These studies suggest that the diabetic-like state during pregnancy is not ascribable to either increased secretion of proinsulin, nor increased rate of insulin degradation.

1.3.2.2. Peripheral Insulin Resistance

Pregnancy is accompanied by developing peripheral insulin resistance as demonstrated by a decreased response to IV administration of insulin (Burt and Davidson, 1974). Most investigators attribute many of the changes in carbohydrate metabolism which occur during pregnancy to reduced tissue sensitivity to insulin.

Proposed mechanisms to explain the peripheral insulin resistance which occurs during pregnancy include decreased insulin receptor binding, or changes in target cells following binding of insulin, ie a post-receptor deficit. Conflicting results have been reported on the change in insulin receptor binding in normal pregnant women compared to non-pregnant controls. Most studies report that there is increased (Gratacos et al., 1981) or unchanged (Puavilai et al., 1982) insulin binding to monocytes. However, decreased binding to monocytes (Beck-Neilsen et al., 1979) and to adipocytes (Pagano et al., 1980) has also been reported. Differences in results may be explained by the fact that most of these studies are cross sectional in nature. In a study where insulin receptor number was determined in serial samples from pregnant women throughout gestation (Andersen et al., 1986) changes in insulin binding to its receptors was dependent on gestational age. There is a significant increase in binding of insulin to its receptor on adipocytes from week 12 to 24 of gestation which is followed by a significant decrease at week 32 and no further change at 4-9 weeks post-partum. These changes in insulin receptor binding to adipocytes are consistent with the biphasic nature of metabolic changes which occur during pregnancy. There is no information on the changes in insulin receptor binding in skeletal muscle tissue or hepatocytes during pregnancy. Therefore the mechanism of insulin resistance is not clear but most believe that it is not due to an alteration in receptor binding of insulin but rather due to the presence of a yet unidentified post-receptor deficit (eg. Puavila et al., 1982).

1.3.2.3. Anti-insulin Endocrine Factors

It has been suggested that the transient diabetic-like state which occurs in pregnancy is due to the influence of anti-insulin hormones such as cortisol, prolactin (PRL), P_4, estradiol (E_2), placental lactogen (PL). These anti-insulin endocrine factors may act either directly to alter metabolic processes, or indirectly by contributing to the increased insulin resistance which develops during pregnancy. Circulating concentrations of these hormones are elevated during pregnancy and become more manifest as pregnancy advances to term.

a) Glucocorticoids

High levels of glucocorticoids such as cortisol present during late gestation (Challis and Olson, 1988), have been shown to bring about a similar kind of insulin resistance that occurs during pregnancy (Hornnes and Kuhl, 1986). Furthermore, there is a significant positive correlation between the increase from early to mid pregnancy in total and free plasma cortisol concentrations and the corresponding decrease in glucose tolerance (Hornnes and Kuhl, 1986). These data suggest that cortisol may be a factor responsible for the decrease in glucose tolerance which occurs during pregnancy.

b) Prolactin (PRL)

During pregnancy there is a steady five to tenfold rise in plasma PRL levels compared with normal menstruating women PRL concentrations (Boyar et al., 1975). Also patients with PRL-producing tumours have impaired glucose tolerance suggesting that PRL has diabetogenic properties (Landgraf et al., 1977). Together these studies suggest that PRL may be responsible for the developing diabetic-like state during pregnancy. However, this is unlikely to be physiological since Skouby and co-workers (1986) found no correlation between the deterioration of glucose tolerance during normal pregnancy and the PRL concentrations measured at this time.

c) **Placental Lactogen**

The major peptide hormone synthesized by the placenta is placental lactogen (PL). It is a 193 amino acid, single stranded polypeptide with a molecular weight of 21-23,0000 Da which is synthesized within cells of the syncytiotrophoblast (Gluckman, 1986). It has 90% sequence homology with growth hormone and 67% with PRL (Bewley et al., 1972). A peptide with similar structural and functional characteristics has also been isolated from ovine placenta (oPL; Chan et al., 1976). PL is detectable in maternal human plasma by 3 weeks gestation and rises progressively until term (Josimovich et al., 1970). PL has also been demonstrated in amniotic fluid (Berle, 1974) and low levels are present in the fetal plasma (Houghton et al., 1984). Circulating concentrations of PL are determined primarily by placental mass (Boime and Bogulawski, 1974) but are consistently elevated during hypoglycaemia induced by maternal starvation (Fielder et al., 1987). The role that PL plays in inducing the alterations in metabolism during pregnancy is equivocal. Studies have demonstrated that PL administration results in impaired glucose tolerance (Beck and Daughaday, 1967, and Grumbach et al., 1968) while others have found no consistent effect (Beck, 1970). In addition, chronic, but not acute, PL administration increases lipolysis in maternal tissues (Gaspard et al., 1977), promotes glycogen storage in the fetus both by stimulating fetal hepatic glycogenesis directly and by antagonizing the glycogenolytic action of glucagon (Freemark and Handwerger, 1977),increases serum free fatty acids, and increases resistance to insulin-induced hypoglycemia (Gaspard et al., 1977). Despite studies which have demonstrated that PL has a clear lipolytic action *in vitro* (Williamson and Coltart, 1978) and *in vivo* (Grumach et al., 1966), PL has paradoxically been shown to stimulate lipogenesis in isolated human adipocytes (Felber et al., 1972). Therefore the exact role of PL in inducing the changes in metabolism which occur during pregnancy is not clear. It is possible that alterations in fat metabolism

causing increased fat stores in early gestation and increased circulating free fatty acid concentrations in later pregnancy, could be caused by elevated plasma PL concentrations present later in gestation.

d) Progesterone (P_4) and Estrogen (E_2)

Other placenta hormones which are released in increasing amounts during pregnancy and have been implicated in the changes in glucose metabolism are P_4 and E_2. The production of these placental steroids involves the interaction between both the placenta and the fetus. During the first few weeks of pregnancy maternal plasma P_4 is largely derived from the corpus luteum. However, from the fifth to sixth week of gestation onwards maternal plasma P_4 is formed in the placenta from circulating cholesterol present in preformed low-density lipoproteins (LDL; Simpson and MacDonald, 1981), and E_2 is synthesized by the cooperation of both the fetus and placenta (Siiteri and MacDonald, 1966). Plasma levels of the steroids increase progressively throughout gestation to reach daily production rates of 250-900 mg of P_4 and 50-150 mg of E_2 in near term women (Jones, 1989). Agents which have been implicated in the regulation of P_4 synthesis by the placenta are β-adrenergic agonists (Caritis et al., 1983), catecholestrogens (Barnea and Fakih, 1985), chorionic gonadotrophin (Demers et al., 1973), and adrenocorticotrophic hormone (ACTH; Barnea et al., 1986). It has also been suggested that normally placental production of P_4 operates maximally at a rate determined by the supply of cholesterol from the maternal circulation (Simpson and Miller, 1978).

Estrogens are produced in the placenta from C_{19} steroid precursors supplied from the fetal adrenal and liver (Siiteri and MacDonald, 1963). The conversion of C_{19} precursors to estrogens is stimulated by human chorionic gonadotrophin (hCG) acting via the adenylate cyclase system to stimulate placental aromatase and sulphatase enzymes (Barnea et al., 1986). Placental estrogen synthesis is determined mostly by substrate

supply of C_{19} steroids from the fetus (Siiteri and MacDonald, 1963). Interestingly, it has been reported that insulin, albeit at high concentrations, stimulates P_4 and E_2 synthesis in placental explants from normal pregnancies but not in those from women with insulin dependent diabetes (Lavy et al., 1987).

Administration of P_4 and E_2 either separately or in combination to non-pregnant adults results in the reproduction of pregnancy associated changes in the structure of the pancreas (Aerts et al., 1980), ultrastructure of the β-cell (Aerts et al., 1980), circulating insulin and glucose levels, and oral glucose tolerance test (Bender and Chickering, 1985). In addition, P_4 and E_2 treatment induces an increase in the plasma insulin:glucagon ratio and in insulin resistance of non-adipose soft tissue (Baird, 1986). In general, the metabolic effects of P_4 and/or E_2 favor a redistribution of the available energy in favour of the fetus at the expense of maternal fat stores. These data suggest that P_4 and/or E_2 are important in mediating the metabolic changes which occur during pregnancy. It is uncertain whether at physiological concentrations in adult animals these steroids act directly on the fat cell or whether the effect on the deposition of fat is exerted solely through the action on insulin secretion. It is also unclear whether the steroids act directly on the pancreatic islets or whether their effect on insulin secretion is achieved solely by increasing resistance to the peripheral action of insulin in non-adipose soft tissue. It is known however, that similar ultrastructural and functional changes in the pancreatic β-cell observed during pregnancy can be reproduced by direct application of P_4 and estrogen to pancreatic islets in culture (Kalkhoff and Kim, 1978; Green et al., 1981; and Neilsen et al., 1986) which suggests that P_4 and/or E_2 can act directly on the pancreatic islet to influence β-cell secretion.

1.3.2.4. Gastrointestinal (GI) Peptides

The available data relating to insulin secretion and degradation in pregnancy clearly point to pregnancy as a state of insulin resistance. In most cases, the insulin resistance of pregnancy is counteracted by increased β-cell mass and sensitivity to glucose stimulation. However, when increases in insulin secretion are insufficient to overcome the developing peripheral resistance. normal glucose tolerance is not maintained and, in severe cases, gestational diabetes may develop. The inability to overcome the developing insulin resistance may be explained by decreased action of potentiators of glucose-stimulated insulin release. Various GI peptides are capable of modulating the ability for glucose to stimulate insulin release (Brown et al., 1978). Very little is known about the effects of pregnancy on the enteroinsular axis. It is known however that basal concentrations of motilin, VIP, somatostatin, and GIP are reduced and the release of these peptides in response to feeding is attenuated during pregnancy (Jenssen et al., 1988). In addition, the physiological incretin effect of the GI tract responsible for accentuating post-prandial insulin release is diminished during pregnancy (Lorrain et al., 1977; Hornnes et al., 1978). These preliminary results suggest that the release of, and the action of, various GI peptides are altered during pregnancy. Therefore it is possible that these peptides may play an important role in inducing the changes in metabolism and may underlie pathological changes which occur in gestational diabetes (Hornnes et al., 1981, 1982, and 1984).

1.4. FETAL METABOLISM

1.4.1. OVERVIEW

1.4.1.1. Amino Acid Metabolism

Amino acids are actively transported by the placenta to the fetus from the maternal compartment (Steel et al., 1979). Circulating concentrations of amino acids in the fetus

are higher than those within the maternal compartment (Girard, 1989). Amino acids present in the fetal circulation are used mainly to synthesize new proteins but evidence exists to suggest that amino acids are also oxidized in the fetus (Krishnamurti and Kitts, 1982). The fetal liver contains enzymes necessary for the oxidation of amino acids and is responsible for a relatively high urea production. It has been suggested that in the ovine fetus catabolism of amino acids can account for approximately 20-25% of fetal oxidative metabolism and can rise to account for as much as 40-50% of oxidative requirements after 72 hours of maternal fasting (Simmons et al., 1974). Normally gluconeogenic pathways are not active in the fetal liver (Warnes et al., 1977; Gleason and Rudolph, 1985a). This is believed to be due to the absence or the very low activity of phosphoenolpyruvate carboxykinase (PEPCK), the rate limiting enzyme of gluconeogenesis (Girard, 1986). PEPCK activity is induced prematurely durinr stressful conditions such as prolonged maternal starvation (Girard et al., 1977; Jones et al., 1984; Lemons et al., 1986). Therefore under adverse conditions the fetus can use amino acids to produce glucose by gluconeogenic pathways.

1.4.1.2. Lipid Metabolism

Fats such as triglycerides, glycerol, and VFA, represent other potential substrates which may also be used by the fetus both as fuels and as carbon sources for growth (Battaglia and Hay, 1984). The transfer of VFA across the placenta has been clearly demonstrated in some species such as rabbits, man and guinea pigs (Hull, 1979) but the placenta is relatively impermeable to fats in other species such as the sheep (Noble et al., 1978) and humans (Dancis et al., 1973). The metabolism of VFA is an important feature of metabolism in ruminants therefore there have been several studies which have attempted to quantitate the relative contributions of the VFA to metabolism in fetal sheep. The data suggest that the transfer of acetate may contribute sufficient carbon to account

for approximately 10-20 % of fetal O_2 consumption and CO_2 production (Comline and Silver, 1976; Char and Creasy, 1976). However, these studies on the relative contributions of the short chain fatty acids in fetal sheep need further extension to conditions of maternal fasting and particularly to an investigation of the question of whether the short chain fatty acids contribute significantly to placental metabolism.

Although a number of studies have suggested that triglycerides may cross the placenta (eg. Thomas and Lowy, 1982), the experimental designs of the studies have not permitted a quantitative estimation of the relative contribution of triglyceride transport as a source of carbon for the developing fetus. It is apparent that glycerol derived from the breakdown of triglycerides is readily transported across the placenta to the fetus, is converted to glucose and glycogen in the fetal liver, and plasma glycerol concentrations increase in pregnant rats during fasting (Battaglia and Hay, 1984; Gilbert, 1977). Therefore it appears that glycerol may be used for endogenous glucose production in the rat fetus and becomes increasingly important to the fetus under conditions of maternal fasting. Adipose tissue is laid down rapidly in fetal sheep between 70 and 120 days gestation (term = 145-150 days) but then develops more slowly until birth (Vernon et al., 1981). The fetus is capable of de novo synthesis of fatty acids from preferably acetate, but also from glucose and lactate (Vernon et al., 1981). Enzymes necessary for fatty acid synthesis are present in adipose tissue of fetal lambs over the last 30 days of gestation and the activity of these enzymes is similar to that present in the adult sheep (Vernon et al., 1981).

There is considerable evidence that placental metabolism plays an important role in providing arachidonic acid to the fetus (Crawford et al., 1976; Battaglia, 1981). However, the extent to which the placenta supplies the fetus with other fats is unknown.

1.4.1.3. Glucose Metabolism

Glucose is believed to be the major substrate utilized by the fetus. It accounts for about 85% of placental oxygen consumption and for about 65% of fetal oxygen consumption, taking into account utilization of lactate produced by the placenta (Hay and Sparks, 1985). Glucose present within the fetal circulation is derived from transplacental flux from the maternal circulation. Transfer of glucose across the placenta is potentially bidirectional depending on the concentration gradient. Since concentrations of glucose in the maternal circulation are normally higher than fetal plasma glucose concentrations, glucose is transported across the placenta from the maternal compartment into the fetal compartment (Hay et al., 1984b). Fetal glucose uptake is directly related to maternal glucose concentration but tends to plateau at glucose concentrations higher than 140 mg/dl, suggesting saturation of a transplacental carrier (Hay et al., 1984b). As a result, below the saturation level, any factor which affects maternal insulin and glucose concentrations, will alter glucose delivery to the placenta and fetus which in turn will affect fetal glucose and insulin concentrations (Fowden, 1985; Schlossman, 1938).

Using tracer methodology and the Fick principle, Hay and co-workers (1984a) have proposed a three pool model to describe transplacental glucose flux, which includes: (1) the maternal compartment, (2) the uteroplacental unit, and (3) the fetal compartment. Seventy-five percent of glucose delivered to the uterus is utilized by the placenta to produce either glycogen or lactate, or it can be metabolized to provide cellular energy for various placental processes. The lactate which is produced by the placenta normally under aerobic conditions, either returns to the maternal circulation or is delivered to the fetal compartment where it is an important source of cellular energy (Gleason et al., 1985). Glucose which is not metabolized by the placenta passes onto the fetus where approximately 60% of fetal glucose is utilized to produce cellular energy (Hay and Sparks,

1985). The remainder of the glucose in the fetal circulation is either converted to glycogen or is used in various anabolic processes which result in the laying down of new tissue.

1.4.2. DEVELOPMENT OF THE FETAL ENDOCRINE PANCREAS

Interest in the endocrine pancreas of the fetus dates from 1920s when Banting and Best first isolated insulin from the pancreas of the fetal calf (Banting and Best, 1922). Since insulin is relatively easy to measure and because of the clinical importance of diabetes, insulin was one of the first hormones to be measured in the unstressed, chronically catheterized fetus and its secretion and function *in utero* have now been investigated in a wide variety of species (Bassett, 1977; Bassett and Thorburn, 1971; Milner et al., 1975). In recent years, there have been more studies on fetal glucagon secretion, but very little is known about somatostatin and PP in the fetus.

Pancreatic endocrine tissue develops from the ducts of the embryonic pancreas and can be distinguished early in gestation in most species (Van Assche and Aerts, 1979). Generally, pancreatic A-cells can be identified using immunocytochemical techniques before the β-cells appear and often single A-cells are found in the periphery of the pancreas during the early stages of pancreatic development. Insulin is present in the human fetal pancreas by 10 weeks gestation (Robb, 1961) and in islet cells of the fetal lamb pancreas by 42 days of gestation (Wiles et al., 1969). Messenger RNA for proinsulin can be isolated in the fetal bovine pancreas in the first trimester (Frazier et al., 1981). Both insulin and glucagon cells show a rapid increase in number during the second phase of proliferation and by term adult type islets of Langerhans can be identified clearly in the fetal pancreas (Bassett, 1977; Pictet and Rutter, 1972). At term, the islets are innervated and have a good blood supply and contain D- and PP-cells in addition to the more common A- and β-cells (Milner, 1980). Endocrine cells containing insulin, glucagon, and

somatostatin are evenly distributed throughout the fetal sheep pancreas whereas PP cells are more numerous in the tail portion compared to the head portion of the pancreas (Pictet and Rutter, 1972). Endocrine cells represent a greater proportion of the total pancreatic tissue in the fetus than the 1% present in the adult (Van Assche, 1979). Of the total amount of endocrine tissue in the fetal pancreas β-cells represent approximately 60%, A-cells 25%, D-cells 10%, and PP cells 5% (Unger and Dobbs, 1978). The pancreatic content of these hormones increases with gestational age probably reflecting the increase in cell number and an enhanced biosynthetic capacity of the individual cells (Bassett, 1977).

Insulin is formed and released from the fetal β-cells in a manner similar to that observed in the adult pancreas (Orci et al., 1971) and is found in the fetal circulation in the same molecular form as in the adult with no significant amounts of proinsulin in the circulation (Bassett and Thorburn, 1971). Insulin and glucagon can be measured in the fetal plasma from at least 42 days of gestation in fetal lambs (Alexander et al., 1968) and at a comparable early stage of development in human fetuses (Adam et al., 1969). Since the placenta has been shown to be impermeable to both insulin and glucagon, these hormones in the fetal circulation are most likely of fetal origin (Alexander et al., 1972). Peripheral insulin concentrations are related linearly to the concentrations in the hepatic portal vein of fetal sheep and therefore these measurements represent a valid estimate of pancreatic insulin secretion in this species. In addition, metabolic clearance rate of insulin is similar in adult and fetal sheep and does not change with gestational age (Fowden, 1980a).

Plasma glucagon concentrations increase with increasing gestational age in a number of species (Fowden, 1985). Studies examining changes in plasma insulin concentrations with gestational age have produced conflicting results (Fiser et al., 1974;

Fowden, 1985; Girard, 1980). This is not unreasonable considering the many factors which are known to influence the secretion of insulin from the fetal endocrine pancreas. Differences may be explained by experimental conditions and times of day when the studies were carried out.

Plasma somatostatin concentrations have yet to be determined in the fetus. However, somatostatin has been shown to inhibit the release of insulin and glucagon in the newborn lamb (Sperling et al., 1977). The physiological significance of these findings to insulin secretion *in utero*, remains to be determined.

PP has been detected in plasma of fetal sheep by d107 of gestation and concentrations increase over the last one-third of gestations to levels much higher than plasma PP concentrations measured in adult sheep (Shulkes and Hardy, 1982). Although PP has insulinotropic properties, a physiological role for PP has yet to be determined in either adult or fetal life.

1.4.3. GLUCAGON IN THE FETUS

Less is known about glucagon secretion *in utero* because of tne difficulties of measuring pancreatic glucagon (Girard and Sperling, 1983). In general, the fetal A-cells appear to respond more readily to neural than the metabolic stimuli (Fowden, 1985). Short term changes in the glucose level have little effect on glucagon concentrations in the fetal lamb and rat (Fowden, 1985). However, prolonged hypoglycemia caused by maternal fasting increases fetal glucagon levels (Girard and Assan, 1981) while hyperglycemia induced by maternal administration of glucose reduces glucagon levels in fetal rats after 72 hours of infusion (Ktorza et al., 1981).

Catecholamines are a potent stimulus to glucagon release *in utero*. Small doses of adrenaline raise glucagon levels in the fetal sheep (Fowden, 1985) and a variety of adverse conditions known to increase endogenous fetal catecholamine levels (Jones et al., 1988) also elevate the circulating glucagon concentrations *in utero* (Girard and Sperling, 1983). Injection of propranolol reduces A-cell response to adrenaline but has little effect on the basal glucagon concentrations. These results suggest that catecholamine-mediated glucagon release is not tonically active *in utero* but is probably important during adverse conditions (Sperling et al., 1980).

Infusion of glucagon into the sheep fetus reduces the umbilical venous-arterial concentration difference of glucose which indicates that the umbilical uptake of glucose is reduced under these circumstances (Phillips et al., 1983). Battaglia (1981) has demonstrated that the specific activity of tracer glucose infused into plasma of fetal sheep is significantly reduced during glucagon infusion. This implies that there is a dilution of the fetal glucose pool by non-tracer glucose presumably reflecting endogenous production of glucose by the fetus. The plasma concentration of glucagon at which fetal glucose production occurred was higher than concentrations of glucagon which occur endogenously but similar to the values observed under adverse conditions (Shelley and Girard, 1981). Hence glucagon is likely to be important in maintaining glucose concentration in stressful situations.

Glucagon infusion raises the glucose level in the fetal sheep (Bassett, 1977), monkey (Epstein et al., 1977), and rat (Hunter, 1969) but little is known about the effects of glucagon deficiency *in utero*. There has been one reported case of specific glucagon deficiency in a newborn human infant which was associated with profound hypoglycemia (Vidnes and Oyasaeter, 1977). However the hyperglycemia observed after pancreatectomy of the fetal sheep suggests that insulin is the more dominant hormone in the control of glucose levels *in utero* (Fowden et al., 1984).

1.4.4. INSULIN IN THE FETUS

The action of insulin on glucose metabolism in the fetus has been examined extensively. Insulin has been shown to lower fetal blood glucose levels in wide variety of species including sheep (Colwill et al., 1970), pigs (Herbein et al., 1977), monkeys (Mintz et al., 1969), and dogs (Schlossman, 1938). Fetal glucose concentrations decrease readily in response to insulin administration and long-term infusion of insulin produces a sustained reduction in plasma glucose levels in fetal sheep (Colwill et al., 1970). Conversely, hypoinsulinaemia *in utero* whether induced by diabetogenic drugs (alloxan or streptozotocin; Fletcher and Bassett, 1986; Alexander et al., 1971; Brimsmead and Thorburn, 1982), or fetal pancreatectomy (Fowden et al., 1984), leads to an increase in the fetal glucose levels. Fowden and co-workers (1984) demonstrated a close correlation between the endogenous concentrations of insulin and glucose in pancreatectomized and intact, sham operated sheep fetuses which strongly suggests that insulin is a physiological regulator of plasma glucose levels *in utero*.

It appears that the hypoglycaemic action of insulin *in utero* is due to enhanced glucose uptake by the fetal tissues. The reduction in fetal glucose concentrations following insulin administration increases the transplacental gradient for glucose and leads to an increase in the transfer of glucose from the mother to the fetus (Simmons et al., 1978). Direct measurements made in fetal sheep have revealed that glucose utilization by fetal tissues doubles during insulin infusion. Since the total rate of oxygen consumption has been shown to remain relatively constant in response to hyperinsulinaemia, insulin would appear to enhance the oxidation of glucose in preference to other metabolites available to the fetal sheep (Hay et al., 1984).

Ablation of normal fetal β-cell function by fetal pancreatectomy or streptozotocin reduces glucose utilization and the umbilical glucose uptake in the sheep fetus (Fowden

et al., 1985). Fetal glucose uptake appears to vary linearly over the range of concentrations observed *in utero* (0-30 µmol/kg.min), but plateaus at a value of about 50 µmol/kg.min (Fowden, 1985). This suggests that there is a maximum rate at which the various biochemical pathways can utilize glucose *in utero*. There also appears to be a minimum rate of glucose uptake by the fetus which presumably represents the minimum rate of glucose utilization by the insulin insensitive tissues.

The role of insulin in fetal metabolism is not confined to glucose homeostasis alone. The concentration of the amino acids, free fatty acids, and other carbohydrates in the fetal circulation are also increased after fetal pancreatectomy (Fowden et al., 1984). Conversely, insulin infusion to the fetal rhesus monkey induces a fall in plasma free fatty acids (Chez et al., 1970) and enhances hepatic enzyme activity (McCormack et al., 1979). Similar results are reported in the hyperinsulinaemic rat fetus in which increased incorporation of labelled fatty acids into the fetal carcass and liver but not lung, brain and placenta occurred (Vileisis and Oh, 1983). There are several reports of increased adipose tissue in the hyperinsulinaemic fetus in both the rat (Picon, 1967) and human (Osler and Pederson, 1960; Fee and Weil, 1963). These studies suggest that the role of insulin in fetal fat metabolism is to stimulate the storage of fats in adipose tissue.

Insulin does not appear to affect human placental amino acid transfer (Steel et al., 1979) or to alter fetal amino acid uptake in the fetal lamb (Young et al., 1977). Other studies have reported that free amino acid tissue pools were reduced following insulin administration suggesting that tissue uptake of amino acid was induced (Gluckman, 1986). Thus, the role of insulin in amino acid metabolism in the fetus has not fully been worked out.

Overall, the anabolic effect of insulin on the metabolic balance of the fetus tends to enhance the growth rate *in utero* by making more substrates, primarily glucose,

available to the fetus for tissue accretion as well as oxidation. Insulin administration has been shown to increase the birth weight of fetal rats (Picon, 1967), rabbits (Fletcher et al., 1982), and monkeys (Susa et al., 1979) at delivery. The enhanced birth weight of the infant of the diabetic mother has been attributed to the effects of hyperinsulinaemia (Milner, 1980). In contrast, the diabetic infant, the infant with pancreatic agenesis, and the pancreatectomized fetal lamb are small at birth (Fowden et al., 1984). In human fetuses, the hypoinsulinemia-induced reduction in fetal growth rate is not apparent until after 23 weeks gestation, suggesting that insulin becomes a critical factor in the regulation of fetal growth at this age (Milner and Hill, 1984). The positive correlation observed between the fetal plasma insulin levels in late gestation and the delivery weight of individual fetal rabbits and lambs shows that insulin has an important role in the regulation of the fetal growth rate (Fowden et al., 1984).

1.4.5. GLUCOSE-STIMULATED INSULIN RELEASE IN THE FETUS

There is considerable information relating to the regulation of fetal insulin secretion although much of the information has been conflicting. This can probably be attributed to the nature of the experimental approaches used since there has frequently been conflict between *in vivo* and *in vitro* data. *In vitro* studies performed using rat pancreata suggest that the insulin response to glucose is present by 18.5 days post-conception (Kervran and Girard, 1976). Similar studies performed using human fetal pancreatic tissue have yielded conflicting results (Schaeffer et al., 1973; Espinosa et al., 1970; Ashworth et al., 1973). Much of the *in vivo* data were initially obtained from exteriorized animal fetuses. The development of techniques which enabled the implantation of chronic indwelling vascular catheters into mammalian fetuses *in utero*, permitted plasma samples to be obtained from fetuses in which the effects of surgery and anaesthesia were controlled. This was

important in studying factors involved in the regulation of insulin secretion since stress depresses pancreatic endocrine function (Fowden, 1980b). Fowden (1980) has demonstrated in fetal sheep between d125-140 of gestation, the β-cell response to exogenous glucose is relatively rapid with a significant increase in plasma insulin within 5-10 min of raising the fetal glucose concentration. Phillips and co-workers (1978) using a kinetic approach, have demonstrated that ovine fetal insulin levels do vary with the endogenous glucose concentrations when the only factor influencing the fetal glucose level is maternal nutritional status. This group also has demonstrated that in fetal sheep the β-cell sensitivity to glucose is similar to that in the adult however the range of glucose concentrations stimulating insulin release is much lower in fetal than adult sheep corresponding to the lower glucose concentrations present in the fetal circulation. With prolonged glucose infusion, the fetal insulin level continues to rise to a plateau value but does not show the typical biphasic response observed in newborn and adult animals (Phillips et al., 1979). The difference in the fetal pattern of response to a long term glucose infusion has been attributed to a lack of stored insulin and to immaturity of the intracellular mechanisms involved in insulin release in the fetus. This seems unlikely however since pancreatic insulin content is in fact higher in the fetus than adult and tolbutamide which stimulates the release of stored insulin, has been shown to evoke a rapid, early phase of insulin release in the chronically catheterized fetus (Phillips et al., 1979). In the horse, glucose sensitive insulin release appears at 270 days of gestation (Barnes et al., 1979). Thus although these studies using the chronically catheterized fetuses strongly suggest that the late gestation fetal pancreas is responsive to glucose, there remains controversy as to the nature of the fetal insulin response to glucose.

The positive correlations observed between the endogenous concentration of insulin and glucose in the chronically catheterized fetal pigs (Fowden et al., 1982a), sheep

(Phillips et al., 1978), and horses (Barnes et al., 1979) clearly demonstrate that glucose is a physiological regulator of insulin release *in utero*.

1.4.6. FACTORS INFLUENCING FETAL β-CELL SENSITIVITY TO GLUCOSE

The β-cell sensitivity to glucose is influenced by a variety of factors including hormones, neurotransmitters, and metabolic substrates.

1.4.6.1. Metabolites

Fowden (1980b) has demonstrated that the insulin response to glucose in the fetal lamb is related to the initial plasma glucose concentration. This suggests that the sensitivity of the fetal β-cell is set by endogenous glucose levels. This suggestion is compatible with observations that chronic fetal hyperglycaemia accelerates the development of insulin secretory mechanisms so that human (Steinke and Driscoll, 1965) and rhesus monkey (Mintz et al., 1972) newborns of diabetic mothers have a mature insulin response to secretagogues. This precocial development of the pancreatic islet in fetuses exposed to maternal diabetes is not observed if the fetus is an anencephalic (Van Assche et al., 1969). Similar results are reported from studies on decapitated rabbit fetuses (Jack and Milner, 1973). These data suggest that the maturational effect of glucose exposure is dependent on the integrity of the hypothalamic pituitary axis or CNS input. Possible endocrine factors involved in this maturational response will be discussed in the next section.

Amino acids appear to be more potent than glucose in releasing fetal insulin *in vitro* (Milner et al., 1975). *In vivo* studies reveal that certain amino acids such as alanine, arginine, and glycine can stimulate insulin release in the fetus directly and mixtures of amino acids potentiate the β-cell response to exogenous glucose in rat (Milner, 1980), sheep (Fowden, 1980b), and human (Grasso et al., 1980) fetuses. Whether amino acids

are physiologically significant to the regulation of fetal insulin secretion is unknown. Plasma amino acid concentrations are high in the fetal lamb therefore it is possible that amino acids could have tonic effects on β-cell function *in utero* (Battaglia and Meschia, 1978).

In fetal lambs as in adult sheep, short chain fatty acids are potent stimuli of insulin release which is further evidence that pancreatic islets in the fetus are subject to regulation in manners qualitatively similar to those in the adult.

1.4.6.2. Nerves and Circulating Catecholamines

Nerves are present in the fetal pancreas by term in most species and receptors for the various transmitters can be demonstrated in the fetal pancreas even before the innervation is complete (Pictet, 1967; Van Assche and Aerts, 1979). The sympathetic nerves to the pancreas appear to be tonically active as injection of alpha (α)- and β-blockers into the chronically catheterized sheep fetus alters the circulating concentrations of insulin (Sperling et al., 1980). In common with adult animals, adrenergic activation through α-receptors suppress fetal insulin secretion while the β-agonists stimulate insulin release (Sperling et al., 1980).

Less is known about the parasympathetic innervation to the fetal pancreas. Cholinergic receptors are present in the fetal pancreas and acetylcholine has been shown to stimulate insulin release in the fetal rat (deGrasparo et al., 1978). Atropine administration also abolishes the reflex release of insulin in response to suckling in newborn lambs (Porter and Bassett, 1979) and diminishes the β-cell response to glucose in young calves (Bloom and Edwards, 1981). These observations suggest that the parasympathetic innervation to the pancreas is functional during the neonatal period and that it may be involved in mediating the effects of the metabolic stimuli.

The infusion of adrenaline or noradrenaline lowers circulating plasma insulin concentrations and abolishes the normal fetal insulin response to both glucose and arginine via alpha adrenergic mechanisms in the fetal lamb (Fowden, 1980b). In addition, this inhibitory effect is observed in fetal lambs when endogenous catecholamine concentrations are elevated due to stress caused by anaesthesia, labour, and hypoxia (Jones et al., 1988).

The peptidergic nervous system has been implicated in the regulation of insulin release in the adult. Information on the importance of this component in determining fetal insulin release is totally lacking.

1.4.6.3. Endocrine Factors

Controversy exists as to whether in late gestation the fetal insulin response to glucose changes or matures. Some studies have suggested that the fetal insulin response to glucose is diminished relative to post-natal secretion (Bassett and Thorburn, 1971) and a maturational increase in the response to glucose has been reported in fetal sheep (Fiser et al., 1974) and horses Barnes et al., 1979). Pituitary, and adrenal hormones have been implicated as mediators of this maturational development of the fetal insulin response. This is based on studies which have demonstrated that removal of the pituitary hormones by fetal decapitation or hypophysectomy increases fetal insulin levels and enhances insulin secretion in response to glucose in fetal rabbits (Jack and Milner, 1973), pigs (Martin et al., 1984), and sheep (Fowden et al., 1984). Further, human anencephalics lacking a pituitary also show abnormalities in pancreatic endocrine functions (Van Assche et al., 1969). Infusion of adrenocorticotrophic hormone (ACTH) into decapitated fetal rabbits restores the normal pattern of insulin secretion which suggests that it is the lack of ACTH that is primarily responsible for the changes in β-cell function after decapitation in this species (Jack and Milner, 1973; Fowden et al., 1984). However,

changes in β-cell innervation in the absence of cerebral tissue may also contribute to the abnormalities observed after fetal decapitation. Liggins (1976) reported that the insulin response to the infusion of glucose was greater in sheep fetuses that had received ACTH *in utero* for 4 days, than in saline treated controls. This observation would be consistent with maturation of the fetal pancreatic β-cell response in association with the pre-partum activation of fetal pituitary-adrenal function (Liggins 1976). Thus controversy exists concerning the nature and timing of onset of the fetal insulin response to glucose and concerning the possible maturation of this response during the last one third of pregnancy.

1.4.6.4 GI Peptides

Several immunocytochemical studies have been performed to determine the ontogeny of various GI peptides such as gastrin, PP, glucagon, GIP, somatostatin, motilin, secretin, neurotensin, and VIP, in many different species including pigs (Alumets et al., 1983), humans (Bryant et al., 1982), and sheep (Reddy and Elliot, 1988). Although slight differences occur between species, in general, GI peptides are first detected very early in fetal development and concentrations measured in the fetus increase progressively as pregnancy advances to levels often greater than those measured in the adult. GI peptides also tend to have a wider distribution throughout the fetal GI tract compared to the adult pattern of distribution. Shulkes and Hardy (1982) have demonstrated that gastrin and PP are detectable in the plasma of fetal sheep as early as day 100 of gestation and levels increase substantially over the last one-third of pregnancy. Lichtenberger and co-workers (1981) have also provided evidence to suggest that gastrin detected in the fetal plasma is produced by abomasal and duodenal G cells in the fetus. These studies indicate that GI peptides, in particular gastrin, are quantitatively important during fetal life. Circulating plasma levels of other GI peptides have not yet been determined in fetal sheep.

Various GI peptides including VIP, and gastrin (Attai et al., 1974), have also been localized in the human placenta and a highly purified rat placental extract (mwt 30,000 Da) has been shown to stimulate insulin release in adult rats. and from isolated neonatal rat islets (Sodoyez-Goffaux et al., 1981). The identity of this peptide remains to be elucidated. In addition GIP has been measured within human amniotic fluid (Falluca et al., 1982). GIP-containing cells are present in the human fetus by 10-12 weeks gestation (Bryant et al., 1982) therefore it is possible that GIP present in the amniotic fluid may be derived from fetal sources. However, the placenta has not yet been examined as a site of GIP production and therefore this possibility can not be ruled out.

Although studies determining the distribution of various GI peptides using immunocytochemical and immunoassay techniques demonstrate that many GI peptides are present in high quantities in the fetus of several different species, most studies which have examined a functional role for these peptides have been conducted in neonates. The post-natal development of the gastrin-induced gastric acid secretion has been studied extensively in rats (Acker, 1982). From these studies it is apparent that the ability of gastrin and other secretagogues such as acetylcholine to stimulate gastric acid secretion appears to develop co-incidently with the time of weaning (Ikezaki, 1983).

Others studies have examined whether plasma levels of GI hormones are elevated in neonates following feeding. In human newborns plasma levels of various GI peptides with insulinotropic activities including GIP and gastrin are elevated following feeding (Lucas et al., 1980a). Similar post-prandial elevations in plasma gastrin concentrations were noted in neonatal swine and this response was attenuated by neonatal antrectomy (Mozam et al., 1980). Others have failed to demonstrate post-prandial elevations in plasma GI peptides (Rogers et al., 1978). The reasons for these discrepancies may be explained by the time following delivery that the studies were carried out. Lucas and co-

workers (1980b) have demonstrated a progressive development of the post-prandial hormone responses during the first few days of life in human fetuses and suggest that early enteral feeding may trigger this post-natal maturation. The responses of GIP and insulin to intraduodenal and IV glucose administration have been compared in order to assess whether the incretin system is present in newborn goats (Nilssen et al., 1983), pigs (Kuhl et al., 1982), and humans (King et al., 1989). It appears from these studies that the ability of GIP to augment glucose-stimulated insulin release is not present in the early neonatal period in goats or pigs but is present in full term newborn infants. At what stage of gestation this incretin effect develops in human fetuses remains to be determined. Further, whether or not the insulinotrophic effect of GIP is present at birth, or develops *in utero*, in other species also remains to be determined.

Little information is known about the effect of GI peptides in fetal sheep. The role of gastrin in the growth and development of the GI tract of fetal sheep has been investigated by Shulkes and his colleagues (1984 & 1985). They have demonstrated that gastrin is not only present in high quantities in the plasma but is also detectable in gastric juice of fetal sheep (Shulkes et al., 1984). Since fetal sheep are hypochlorhydric until very late in gestation, gastrin does not undergo peptic degradation and therefore may exert effects locally on the gastric mucosa in a paracrine manner (Shulkes et al., 1985). Gastrin is known to possess trophic actions in the adult (Johnson, 1976) therefore it is tempting to suggest that gastrin, present in the fetal gastric juice and plasma, plays a role in the growth and development of the GI tract *in utero*. However, Trahair and Harding (1987) have demonstrated that fetal antrectomy which reduces circulating gastrin concentrations, resulted in little change to various morphometric parameters measured in the GI mucosa. This lack of response to the removal of endogenous gastrin may be explained by the presence of extra-gastric sites of gastrin production such as the duodenum and pancreas

(Track et al., 1979). It is also possible that fetal antrectomy was not performed at a stage in gestation critical for gastrin-induced GI development.

2. RATIONALE, HYPOTHESIS, AND STATEMENT OF OBJECTIVES

2.1. RATIONALE

Despite the fact that the first suggestion that some factor present in the GI tract was capable of augmenting glucose-stimulated insulin release was made in the late 1800's, studies examining the nature of this factor(s) have only occurred within the last twenty years. From these studies it is apparent that the effects of nutrients (glucose, amino acids, and fats) and their metabolites on the pancreas cannot account adequately for the regulation of the endocrine secretions of the pancreas. GRP has been demonstrated to induce insulin secretion and to augment glucose-stimulated insulin release in several monogastric species including dogs (McDonald et al., 1981), mice (Pettersson and Ahren, 1987), and pigs (Knuhtsen et al., 1987), and to augment glucose-stimulated insulin release in rats (Greeley and Thompson, 1986; Martindale et al., 1982) and in man (Wood et al., 1983). Bloom and co-workers (1984) have found similar results in conscious calves.

Very little is known about the effect of GI peptides in the fetus. Numerous immunocytochemical studies have demonstrated many of these peptides are quantitatively important in fetal life (eg. Shulkes and Hardy, 1982) and it has been suggested that they are important in directing the proper growth and development of the fetal GI tract. Additionally, insulin is very important in determining proper fetal growth (Fowden, 1989; Milner and Hill, 1984). Factors regulating insulin secretion from the fetal pancreas are therefore of interest. The effect of GI peptides on pancreatic endocrine secretions in the fetus has not previously been examined in any species. The development of techniques which enabled the implantation of chronic indwelling vascular catheters into mammalian fetuses *in utero*, permitted plasma samples to be obtained from fetuses in which the effects of surgery and anaesthesia are controlled. This was important in studying factors

involved in the regulation of insulin secretion since stress depresses pancreatic endocrine function (Fowden, 1980b; Jones et al., 1988). Thus, the chronically catheterized fetal sheep represents a good animal model to examine whether factors, such as GRP, influence insulin release during fetal life and whether the action of these agents changes or matures over the last one third of gestation. However. the effect of GRP on the endocrine pancreas of adult sheep must first be determined and compared to responses which occur in other species.

Pregnancy is known to be associated with marked changes in carbohydrate, fat and protein metabolism. Metabolic changes such as increased peripheral insulin resistance, are important in facilitating appropriate delivery of nutrients to the fetus and hence proper fetal growth and development. One consequence of the metabolic changes during pregnancy is impaired glucose tolerance which becomes progressively worse as pregnancy advances to term (Baird, 1986) and can result in the development of gestational diabetes (Hornnes et al., 1978). Many mechanisms underlying the development of glucose intolerance during pregnancy have been proposed and investigated. Hornnes and co-workers (1978) have proposed that normal glucose tolerance is not maintained because increased insulin secretion during pregnancy is not sufficient to compensate for the concurrent development of peripheral insulin resistance which occurs. This inability to overcome the peripheral insulin resistance may be explained by decreased action of potentiators of insulin release. GRP is known to modulate glucose-stimulated insulin release in non-pregnant adult humans (Wood et al., 1983) and rats (Greeley and Thompson, 1984; Martindale et al., 1982). The action of GRP on the maternal endocrine pancreas has not been examined previously in any species. Whether the ability of GRP to stimulate insulin release is also glucose dependent in sheep remains to be determined. Also whether the ability of GRP to induce insulin

secretion and augment glucose-stimulated insulin release is less in pregnant compared to non-pregnant sheep is also not known. Factors which are responsible for mediating any changes during pregnancy also need to be investigated. How the actions of GRP on the endocrine pancreas within the maternal compartment alters fetal and glucose and insulin concentration must also be determined.

Although GRP stimulates insulin release in all species examined to date, species differences exist with respect to the mechanism by which GRP exerts its actions on the endocrine pancreas. In monogastric species it appears that GRP is localized within the GI tract and pancreas exclusively within nerves and these nerves have been localized closely associated with pancreatic islet cells (Ghatei et al., 1984; Greeley and Thompson, 1984). In these species it therefore appears that GRP is released from local nerves following post-ganglionic parasympathetic stimulation (Knuhtsen et al., 1984) and can act directly to stimulate insulin release from the β-cell. In contrast to monogastric animals, GRP is not detectable in the pancreas but is present in neural structures of the GI tract and in the systemic circulation of rumminants (Bloom et al., 1984; Dockray et al., 1979). Further, plasma GRP elevations are observed in calves following splanchnic (Bloom et al., 1980), but not vagal (Adrian et al., 1983), nerve stimulation. Bloom and his colleagues (1984) have also suggested based on preliminary studies, that feeding of calves which is associated with increased sympathetic discharge (Bloom et al., 1978), produced significant increases in circulating plasma BLI. These findings suggest that in ruminants GRP is released under physiological conditions from post-ganglionic sympathetic nerves into the systemic circulation and acts in an endocrine fashion to stimulate insulin release from the pancreas. Whether GRP exerts its insulinotropic effects in a similar way in other ruminant species such as the sheep remains to be determined.

2.2. HYPOTHESIS

These studies examine the overall hypothesis that effects of gastrin releasing peptide (GRP) lead to changes in plasma insulin and glucose concentrations in fetal sheep. This could occur due to actions of GRP directly within the fetal compartment or due to indirect effects of GRP on glucose and insulin concentrations in the maternal compartment which in turn influence the availability of glucose for transplacental transfer to the fetus and continued fetal growth.

2.3. SPECIFIC OBJECTIVES

(1) To examine if exogenous glucose administration stimulates insulin release in fetal and adult sheep and determine whether the fetal insulin response to glucose changes over the last one-third of gestation.

(2) To determine pancreatic endocrine responses to exogenous administration of GRP in adult non-pregnant sheep.

(3) To determine if the insulinotropic effect of GRP is present in fetal sheep and whether this response changes over the last one-third of gestation.

(4) To determine the effect of GRP on pancreatic endocrine secretions in pregnant sheep in late gestation, and to compare this response to that observed in adult non-pregnant sheep.

(5) To determine if the insulinotropic effect of GRP is dependent on circulating glucose concentrations in fetal, pregnant, and non-pregnant sheep.

(6) To determine if the ability of GRP to induce insulin secretion and augment glucose-stimulated insulin release is modulated by circulating P_4 and/or E_2 concentrations.

(7) To determine the distribution of GRP within fetal, adult non-pregnant, and pregnant sheep and suggest possible modes of delivery by which GRP exerts its insulinotropic effect in these animals.

3. GENERAL METHODS

3.1. DATED PREGNANT SHEEP

Mixed breed non-pregnant and time-mated pregnant sheep were used in these studies. In order to obtain fetuses of a known gestational age at regular intervals throughout the year, ovulation was synchronized by implanting a vaginal sponge containing 60 mg medroxy P_4 acetates (Upjohn, Orangeville, Ont.) into each ewe for 14 days. The sponge was then removed and the ewe injected with pregnant mare serum gonadotrophin (PMSG; Synkron Corp., Victoriaville, Ont.) Two days following sponge removal and PMSG injection the ewe was placed with a ram equipped with a brisket harness to establish the time of mating which was then taken as day 0 of pregnancy, unless the ewe returned in heat at a later date.

3.2. SURGICAL PROCEDURES

Surgery was performed between day 100-125 of gestation with term equal to a approximately 145 days in this species. Ewes were fasted for 36-48 hr before surgery. General anaesthesia was induced with 40 ml of 2.5% sodium pentothal (Abbot Laboratories, Montreal, Canada) injected intravenously into the ewe via venipuncture. Anaesthesia was maintained following tracheal intubation with 1.5-2.5 % halothane (CDMV Inc. St Hycinthe, Que.), and a 50:50 mixture of nitrous oxide and oxygen at a flow rate of 2-3 l/minute. The pre-operative procedures consisted of clipping the abdominal area and scrubbing it with an iodine soap. Following transfer of the animal into the operating room, an iodine/alcohol solution was applied to the surgical field.

Surgery was performed using standard sterile techniques. The uterus was exposed through a lower abdominal mid line incision. One fetus per ewe was catheterized. The catheters were filled with heparinized saline and the catheter volume

was recorded as the catheter dead space. In experiments where an acute glucose challenge was administered, the fetal hind limb was exteriorized and the vascular catheters were placed in the femoral artery and vein. In all other experiments the head of the fetus was exteriorized at the time of surgery and the carotid artery and jugular vein were catheterized. Once the fetus was exteriorized, babcock clamps were used to secure the edges of the uterine incision to the fetal trunk distal to the umbilicus in order to prevent loss of amniotic fluid and/or exposure of the umbilical cord. The portion of the catheter remaining outside of the fetus was sutured loosely to the skin in two places. An open ended amniotic fluid catheter was sutured to the outer surface of the fetus. The fetus was returned to the uterus and the uterus was closed subsequently. The maternal abdomen was closed in layers so as to prevent herniation. All catheters were exteriorized through a trocar puncture in the flank of the ewe and the wound was closed with a purse-string suture. Catheters were also placed in the maternal femoral artery and vein and advanced 20 cm to lie in the maternal abdominal aorta and inferior vena cava, respectively. Post-operatively the ewe was given a single intramuscular injection of 75 mg of Banamine (Flunixin, 50 mg/ml; Schering Canada Inc., Pont Claire, Que.) for analgesia.

3.3. POST-OPERATIVE CARE

During the seven day post-surgery recovery period, catheters were filled and flushed daily with sterile heparinized saline (1:500, v/v; 10 USP/ml; 0.9 % sodium chloride). In order to assess fetal health, a 0.5 ml fetal arterial blood sample was collected daily and fetal blood pH, pCO_2, and pO_2 were determined using a acid base blood gas analyzer (ABL-3, Radiometer, Copenhagen). At the time of surgery and for three days thereafter, 4 ml of Pen-di-strep (200,000 IU Penicillin G and 250 mg dihydrostreptomycin/ml; Rogar, London, Ont.) was given by intramuscular injection to the

ewe. In addition, I ml Crystapen (1,000,000 IU Penicillin G; Ayerst, Montreal, Que.) was administered to both the fetal vein and the amniotic cavity over the same time period. Catheters were flushed daily between experimental periods to maintain their patency.

Continuous recordings of intrauterine pressure were made beginning on d140 of gestation and carried on until the ewe entered spontaneous labour as judged by changes in the recordings (Lye et al., 1985). Pressure was recorded from the amniotic fluid catheter using a previously sterilized Statham pressure transducers (Model P-23 ID, Gould, Oxnard, CA.). Recordings were made with a Grass DC amplifier (Model 7 P122C, Grass Instruments, Quincy, Mass.) on a 16 channel Grass Polygraph (Model 78D, Grass Instruments, Quincy, Mass.).

From the day of surgery until euthanasia at the onset of labour, the sheep were housed in individual metabolism cages. These cages allow the ewe to move back and forth and lie down at will. The animal housing room was maintained on a 14 hour light (0600 to 2000 hours) and 10 hour dark cycle. Every morning around 0800 hr the animal housing rooms and cages were cleaned and the ewe received fresh water, hay, and 0.4 kg of grain concentrate (14% Ewe Fitting and Nursing Chow, Masterfeeds, London, Ont.; see Appendix II for food composition). The ewes were provided with similar amounts of hay and grain around 1500 hr each day.

3.4. BLOOD SAMPLING

To obtain fetal and maternal arterial blood samples, a previously heparinized syringe was attached to the stopcock and a volume of blood equal to twice the dead space of the catheter (previously determined at the time of surgery) was drawn into and retained within the syringe. The blood sample was then collected into a second heparinized syringe which had been also been placed on the stopcock. Following the

sample, the blood that was in the catheter was returned and a further amount of heparinized saline equal to the volume of the blood sample removed, was flushed into the catheter. Arterial samples, once obtained, were immediately transferred to ice cold polystyrene tubes and kept on ice until centrifugation. Samples were centrifuged as quickly as possible, within 30 min, at 1500 x g for 10 min at 4 C, following which the plasma was removed and separated into aliquots and stored at -20 C until subsequent analysis.

3.5. PLASMA GLUCOSE ANALYSIS

Plasma glucose concentrations were determined using a Beckman Glucose Analyzer II (Beckman, Palo Alto, CA). This instrument measures the rate of oxygen consumption after addition of the sample to a buffered solution containing glucose oxidase. This glucose oxidase technique specifically measures β-D-glucose and not other glucose isomers such as L-glucose. The minimum sensitivity of this instrument is 5 mg/dl. Aliquots of 10 µl of heparinized plasma were used to determine plasma glucose concentrations and measurements were repeated for each sample until two replicates within a range of 3 mg/dl were obtained. The average of these replicates was then calculated and recorded as the plasma glucose concentration for that sample. Intra-estimate variation was 10.4%.

3.6. RADIOIMMUNOASSAYS (RIA)

3.6.1. Insulin RIA

Immunoreactive insulin was measured in 0.1 ml aliquots of plasma using a RIA performed as described previously (Brown et al., 1975). Samples were thawed only once. The primary antibody used (final dilution = 1:500,000, obtained from Dr. Peter Wright,

Cambridge, England) was raised in rabbits against human insulin and does not cross react with GRP, Galanin, GIP, PP, or pancreatic glucagon. Ovine insulin (Novo Biolabs, Willowdale, Ont.) was serially diluted to obtain 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 µU/ml assay standards which were used to generate a standard curve. The mean assay sensitivity, assessed as three times the standard deviation of bound counts in the absence of cold insulin, was 1.4 µU/ml. Samples with less than 1.4 µU/ml were ascribed the minimum detectable dose for that assay. Intra- and inter-assay coefficients of variation were 3.6 % and 9.5 % respectively.

3.6.2. Glucagon RIA

Plasma immunoreactive glucagon concentrations were determined by a RIA method as described previously (Brown et al., 1975). The primary glucagon antibody (30K; final dilution = 1:500,000, obtained from Dr. R. H. Unger, Dallas, Texas) recognizes the 18-29 glucagon fragment but not the 1-26 fragment of glucagon. The C-terminally directed antibody therefore cross reacts 100% with all of the immunoreactive glucagon moieties present in the plasma. It does not cross react with GIP, secretin, gastrin, VIP or insulin. The mean assay sensitivity was 28 ± 9 pg/ml. Samples with less than the sensitivity of the assay were ascribed the minimum detectable dose for that assay. The intra- and inter-assay coefficients of variation were 6.3 % and 19.0% respectively.

3.6.3. Progesterone and Estradiol RIA

The concentration of P_4 and E_2 were determined in heparinized plasma collected from non- pregnant and pregnant sheep using RIA techniques described previously (Erickson, Challis, and Ryan, 1974). Briefly, aliquots of 0.5 to 1 0 ml of the plasma samples were extracted using 5 ml of diethyl ether. The aqueous phase was separated from the organic phase by rapid freezing. The organic phase was collected and dried down. Samples were reconstituted using phosphate buffered saline and a 0.1 ml and 0.4 ml aliquots were used in the P_4 and estrogen assay, respectively.

The concentration of P_4 was determined in the plasma extract using a RIA previously validated in this laboratory (Erickson, Challis and Ryan, 1974) using an antisera (kindly donated by Dr. D. T. Armstrong, London, Canada) whose cross reactivities have been described previously (Glickman, Carson, and Challis, 1979). In brief, P_4 antibody cross reacts 25-100 % with other C_{21}-steroids and less than 0.1% with cholesterol and C_{18}-steroids. The minimum detectable P_4 concentration in this assay was 2.6 ± 0.3 pg/ml. Intra- and inter-assay coefficient of variation for the P_4 assay were 15.2% and 17.8%, respectively.

Plasma E_2 concentrations were determined by RIA techniques using an antiserum with cross reactivities previously described (Challis and Patrick, 1981). In brief, the estradiol antibody cross reacts 100% with estrone, 0.1-3% with other C_{18}-steroids, and less than 0.1% with other C_{21}- and C_{18}-steroids. The limit of sensitivity of the assay was 2.5 ± 1.1 pg/ml of E_2. Intra- and inter-assay coefficients of variation for the E_2 assay were 9.4% and 9.8% respectively.

3.7. DATA ANALYSIS

Data analysis for each set of results is described in the method section of the chapter in which the data are presented.

4. INSULIN RESPONSE TO GLUCOSE IN FETAL SHEEP

4.1. INTRODUCTION

Before examining the insulin response to GRP in the fetus it was important to first determine if the fetal endocrine pancreas responded to exogenous glucose administration. Controversy exists regarding the nature and time of onset of the insulin response to glucose in fetal sheep. Several early studies reported that the concentration of insulin in the plasma of the fetal lamb rose slowly and only to a small extent in response to a glucose challenge (Wiles et al., l969; Bassett and Thorburn l971). As a result of these studies, the fetal insulin response has been described as sluggish and immature. Other studies using the chronically catheterized fetal lamb have questioned whether the fetal pancreas is relatively unresponsive to glucose during late gestation. Phillips et al. (l978) showed that the insulin sensitivity to glucose, as measured by the slope of the insulin response curves, was similar in fetal and adult sheep and that there was a close correlation between the concentrations of insulin and glucose in fetal plasma before, during, and after maternal fasting. Fowden (l980) demonstrated that plasma insulin concentrations rose rapidly in response to an acute glucose load administered to fetal sheep as early as d124 of gestation. There is limited evidence that a fetal insulin response to a glucose load is present before day 100 of gestation (term 145-150 days) based on observations on one (Bassett and Thorburn l971) and two (Phillips et al. l978) fetuses. These findings argue that insulin has a role in glucose homeostasis in the fetus and that an insulin response to glucose is present in fetal sheep by at least d124 of gestation. However, the earliest gestational age at which insulin is released from the endocrine pancreas of fetal sheep in response to a glucose challenge, is not known.

It is unclear also whether the magnitude of the fetal insulin response changes during late gestation. Fowden (1980a) reported that the fetal insulin response to exogenous glucose was not related to gestational age but she studied ovine fetuses only between d124 and d140 of gestation. Others have reported that the insulin response to exogenous glucose increases in the fetal sheep (Fiser et al. 1973) and horse (Barnes et al 1979). Liggins (1976) reported that the insulin response to the infusion of glucose was greater in sheep fetuses that had received ACTH *in utero* for 4 days, than in saline treated controls. This observation would be consistent with maturation of the fetal pancreatic β-cell response in association with the pre-partum activation of fetal pituitary-adrenal function (Liggins 1976). Thus controversy exists concerning the nature and timing of onset of the fetal insulin response to glucose and concerning the possible maturation of this response during the last one third of pregnancy.

The purpose of this series of experiments was to examine whether the fetal insulin response to glucose was present by d110 of gestation (an earlier gestational age than has been tested previously) and whether this response changed or matured in fetuses tested between d110-d145 (term). The insulin response to an acute glucose load was also examined in adult non-pregnant sheep and compared to that observed in fetal sheep over the last one third of gestation.

The following specific questions were addressed: (1) Is the fetal insulin response to glucose present by day 110 of gestation? (2) Does the fetal insulin response to glucose change or mature between d110-d145 of gestation? (3) How does the fetal insulin response to glucose compare to that observed in adult sheep? and (4) How does IV glucose administration to the fetus affect maternal insulin and glucose concentrations?

4.2. MATERIALS AND METHODS

4.2.1. Animals

Nine mixed breed pregnant sheep of known single insemination dates were used in this first preliminary study. Surgery was performed on five animals between d100-105 of gestation and on four animals around d120 of gestation. Under general anaesthesia, vinyl catheters were implanted into the fetal and maternal femoral artery and femoral vein, and amniotic cavity using the techniques described in section 3.2. Post-operative care was carried out for at least seven days as described previously in section 3.3.

Studies were also performed in adult non-pregnant sheep in which catheters had been surgically introduced into the femoral artery and vein.

4.2.2. Basal Glucose and Insulin Concentrations

Fetal and maternal arterial samples (0.5 - 1.5 ml) were collected daily for determination of basal glucose and insulin concentrations between 1200 and 1600 hr which was 4 to 8 hr after feeding. No samples collected within 7 days of surgery were included. On days when glucose challenge experiments were performed basal glucose and insulin concentrations were determined using the pre-injection samples. The average plasma glucose and insulin concentrations for individual animals were calculated for those samples collected between d110-115, d125-d130, d135-140, and d140-145 of gestation.

4.2.3. Glucose Challenge Experiments

Experiments were performed in healthy fetal sheep (pO_2 > 20mmHg, pCO_2 = 40-50mmHg, pH = 7.30-7.35) at four stages of gestation: d110-115 (n=5), d125-130 (n=5), d135-139 (n=5), and d140-145 (n=4). Experiments were performed between 1200 and 1600 on successive days in a randomized order. Certain fetuses were studied at more than one gestational age. Glucose (0.8g glucose/kg and 1.6 g glucose/kg) prepared in 10 ml of saline or an equal volume of the vehicle, was administered to the fetuses on

separate days in the three later gestational age groups (d125-130, d135-139, d140-145). The younger fetuses (d110-115) received only the lower amount of glucose (0.8g/kg). Doses of glucose administered to fetuses were based on estimated fetal body weights of 1.5 kg at d110-115, 2.25 kg at d125-130, 3.0 kg at d135-139, and 3.5 kg at d140-145 (Barcroft, 1945). In preliminary studies, two fetal lambs between d125-130 of gestation received 1.6 g/kg estimated fetal body weight of L-glucose.

The test solutions were administered as a bolus injection over 15-20 sec via the fetal femoral venous catheter and the catheter was flushed with a further 2 ml of saline. Fetal arterial blood samples (1.0 ml on d110-115 and 1.5 ml at other ages) and maternal arterial blood samples (4.0 ml) were collected at -30, -1, +10, +20, +30, +60, +120 min relative to the injections and processed as described previously (section 3.4).

Similar experiments were performed on the five adult non-pregnant sheep. Saline (20 ml) or an equal volume of glucose (0.8g/kg body weight) dissolved in saline, was administered via the femoral vein. Arterial blood samples were collected before, during and after the injection of the test substance at similar times described above and processed as described previously (section 3.4).

Plasma insulin concentrations were measured in all plasma samples collected from fetal, pregnant and non-pregnant sheep, using a RIA performed as described in the previous section (section 3.6). Plasma glucose concentrations were also determined in all samples taken using techniques described previously (section 3.5).

4.2.4. Data Analysis

Results are presented as the mean ± SEM for the number of animals indicated (n). The effect of time and treatment on glucose and insulin concentrations in plasma was assessed using a nested analysis of variance where numbers in treatment groups were equal. and using a two way unbalanced analysis of variance where there were unequal

numbers between treatment groups. Multiple comparisons were then performed using Duncan Multiple Range test. The relationship of change in basal and peak glucose and insulin concentrations as a function of gestational age was tested using an orthogonal polynomial test. Changes in plasma insulin and glucose concentration after glucose injection were calculated as the summed increases over the mean of the two pre-injection values for each individual fetus. One way analysis of variance was used to test significant differences between treatment groups at each gestational age. A value of $p<0.05$ was considered statistically significant.

4.3. RESULTS

4.3.1. Basal Glucose and Insulin Concentrations

The basal concentration of glucose in fetal plasma was 8 ± 4 mg/dl between d110-115 of gestation and increased linearly with respect to gestational age, to become significantly greater in fetuses between d140 and d145 of gestation (20 ± 3 mg/dl, $p<0.05$; Fig. 1). Basal glucose concentrations in fetal plasma were significantly ($p<0.05$) lower than basal glucose measured in adult non-pregnant sheep and pregnant sheep at any of the gestational ages examined. Maternal basal glucose concentrations tended to increase between d110 and d139 of gestation and then decrease close to term (d140-145). Mean basal glucose concentrations measured in pregnant sheep between d135-140 of gestation (68 ± 3 mg/dl) were greater than those measured in adult non-pregnant sheep (63 ± 5 mg/dl). However, basal glucose concentrations measured in the maternal circulation were not related statistically to gestational age and were not significantly different from adult non-pregnant sheep.

Despite the differences observed in the basal glucose concentrations in fetal plasma, the basal concentrations of insulin in fetal plasma were not significantly different between gestational ages (Fig. 1). The mean basal insulin concentrations measured in fetal sheep between d135 to d139 (6 ± 2 μU/ml) of gestation were significantly less than basal insulin concentrations in adult pregnant sheep (12 ± 3 μU/ml at d110 -115; $p<0.05$), and non-pregnant sheep (11 ± 3 μU/m; $p<0.05$). However, no statistical difference was present between basal insulin concentrations in fetal sheep at any other gestational ages compared to those measured in adult pregnant or non-pregnant sheep.

4.3.2. Fetal Insulin Response to Glucose Administration

There was no demonstrable change in fetal plasma glucose or insulin concentrations following administration of L-glucose into two fetal sheep between d125 and 130 of gestation (Fig. 2). Furthermore, there was no significant change in fetal or maternal plasma glucose or insulin concentrations following a bolus injection of saline into fetal sheep at any gestational age tested (Fig. 3).

Between d110 and 115 of gestation the mean peak concentration of glucose in fetal plasma (44 ± 9 mg/dl) occurred at +10 min after the injection of glucose (0.8g/kg, $p<0.05$; Fig. 3a). The concentration of insulin in fetal plasma was significantly elevated above saline treated controls at +10 min ($p<0.05$). It increased further to mean maximum values of 32 ± 5 μU/ml at +20 min ($p<0.05$), and then subsequently fell toward baseline (Fig. 3b).

In fetuses between d125 and d130 of gestation, mean peak glucose concentrations of 60 ± 8 and 94 ± 11 occurred at +10 min after the glucose injection of 0.8 g/kg and 1.6 g/kg, respectively ($p<0.05$; Fig. 3c). Plasma insulin concentrations rose to mean peak values at +20 min of 22 ± 4 and 39 ± 13 μU/ml following 0.8 g/kg and 1.6 g/kg of glucose, respectively which were significantly greater than plasma insulin concentrations measured in saline treated controls ($p<0.05$; Fig. 3d).

Between d135 and d139 of gestation, a bolus injection of glucose caused a rise to mean peak glucose concentrations of 68 ± 6 and 87 ± 4 mg/dl at +10 min for 0.8 and 1.6 g/kg doses respectively, (Fig. 3e). The corresponding mean peak insulin concentration of 28 ± 6 and 44 ± 22 µU/ml occurred between +30 and +60 min post-injection (Fig. 3f) and were significantly greater than saline treated controls ($p<0.05$)

In fetuses tested between d140-145 of gestation, mean peak glucose concentrations of 85 ± 16 and 155 ± 36 occurred at +10 min following the injection of 0.8 and 1.6 g/kg of glucose, respectively (Fig. 3g). The corresponding mean peak concentrations of insulin (44 ± 10 and 62 ± 11 µU/ml) were achieved at +20 min ($p<0.05$; Fig. 3h).

4.3.3. Responses of the Ewe to Glucose Administration to the Fetus

After administration of glucose at a dose of 1.6 g/kg to the fetus, the concentration of glucose in the fetal circulation rose to values in excess of the basal concentration of glucose in the maternal compartment (Fig. 4.). As a result small elevations in maternal plasma glucose concentrations were detectable. However, changes in the mean plasma glucose concentration in four pregnant sheep during glucose administration to the fetus were not statistically significant. Correspondingly, maternal plasma insulin concentrations were also not changed significantly. Peak glucose elevations measured in the plasma of fetal sheep following an injection of 0.8 g/kg of glucose were not greater than maternal basal glucose concentrations (data not shown). Correspondingly, there was no significant change demonstrable in maternal plasma glucose and insulin concentrations after administration of glucose at 0.8 g/kg to fetuses at any stage of pregnancy.

4.3.4. Responses to Glucose in Adult Non-pregnant Sheep

Glucose administration (0.8g/kg) to adult non-pregnant sheep resulted in large elevations in plasma glucose levels to mean peak values of 363 ± 40 at +10 min after the

injection. In successive samples, the concentration of glucose decreased gradually, but remained significantly elevated over pre-injection values even at +120 min following the bolus injection of glucose (Fig. 5; $p<0.05$).

The insulin response to a bolus injection of 0.8 g/kg of glucose occurred rapidly in adult sheep reaching 91 ± 11 μU/ml by +10 min ($p<0.05$). Despite the declining concentrations of glucose, the concentration of insulin rose further to 120 ± 28 μU/ml at +60 min. Corresponding to the elevated plasma glucose concentrations, plasma insulin levels also remained elevated above pre-injection values +120 min following the administration of the glucose challenge.

4.3.5. Comparison of the Insulin Response to Glucose Between Fetal and Adult Non-pregnant Sheep

The changes in glucose and insulin concentrations following glucose administration (0.8g/kg) to fetal and adult sheep are expressed as the mean ± SEM of the percentage of the maximum elevation attained for each individual animal at each sampling time (Fig. 6). Glucose rose similarly in the fetuses as in the adult with peak values occurring in both cases in the first sample taken +10 min following the injection. Glucose levels returned to baseline values more rapidly in the fetus, whereas in the adult the concentration of glucose remained elevated at +120 min. Correspondingly, in the adult sheep, plasma insulin concentrations remained elevated following the injection of glucose; in the fetus the concentration of insulin had returned to pre-injection values by +60 min.

In fetal sheep, there was a significant increase in the peak glucose concentration attained following the bolus injection of glucose (0.8g/kg) as a function of gestational age ($p<0.05$), but these values were always less than those observed in the adult sheep (Fig. 7b).

The peak insulin concentration after glucose administration to fetuses between d125 and d130 of gestation was significantly less than that in fetuses between d140 and d145 of gestation ($p<0.05$), but not significantly different at other times in gestation (d110-115 and d135-139; Fig. 7a). The peak insulin concentration in fetuses of any gestational tested was always significantly less than that achieved in adult sheep. However, the ratio of the insulin released per unit glucose elevation was not significantly different between fetal and adult sheep (Fig. 8).

4.4. DISCUSSION

The administration of an acute glucose challenge elicited insulin release in the fetus at all gestational ages that were tested (d110-145). There was a significant increase in the magnitude of the peak insulin concentrations attained in fetuses between d125 and d140 of gestation, although values never reached those seen in non-pregnant adult animals. This may reflect, at least in part, the higher peak glucose concentration achieved with the same glucose bolus in the fetus during late gestation and with the further increase in peak glucose levels attained in adult sheep. In fetuses, the ratio of insulin released per unit glucose elevation did not change significantly in late gestation and was not statistically different from the adult.

In these experiments we have estimated fetal body weight based on published charts (Barcroft 1945). In calculating injection doses, we have not included placental weight, although it remains relatively constant in late gestation in the sheep (Barcroft 1946). If the placenta is a major site of glucose utilization, its contribution relative to that of the fetus would be greater in those animals tested at earlier gestational ages. The progressive rise in peak glucose in older fetuses might reflect the change in fetal/placental weight ratio that occurs with the progression of pregnancy.

All experiments were conducted after a post-operative recovery period of at least 7 days. Shelley (1973) showed that fetal plasma glucose and insulin concentrations had stabilized by 5-6 days after surgery. Experiments were also conducted at the same time of day to minimize any effects of diurnal rhythms of the fetal endocrine environment on the parameters that we have measured. The relative constancy of the glucose concentrations in the fetal plasma in control animals, which were in the range of values for basal glucose that have been reported previously (Hay et al. 1984a, 1984b), suggests that the fetuses were in a metabolic steady state. In this study, basal glucose concentrations in the fetus rose progressively throughout late gestation. There was a similar general increase in maternal basal glucose concentrations between d110 and d139 of gestation, consistent with previous observations by Shelley (1973). In that study, samples obtained within 2 days of delivery were excluded from statistical analysis. Maternal glucose concentrations decreased close to term (d140-145) perhaps reflecting voluntary reduction in food intake by the mother, which occurs pre-partum. Variable results of changes in basal fetal glucose concentrations in relation to gestational age reported by other investigators (Shelley 1973) could possibly be explained by differences in sampling times or the state of the fetus after surgery.

Experiments were performed on more than one occasion in certain animals. However, there was no difference in the insulin response to glucose in fetuses that had previously been exposed to a glucose challenge, compared with animals in which the glucose challenge tests were conducted for the first time. Similar observations have been made in other studies (Fowden 1980a). It is unlikely that our findings represent changes in the half-life of insulin, since the metabolic clearance rate of insulin does not change in the fetus over the last third of pregnancy nor between fetal and adult sheep (Fowden 1980a).

The present experiments examined the insulinotrophic effects of glucose. Amino acids (Fowden, 1980b), fatty acids (Bassett et al., 1983) and fructose (Davis, et al., 1970) are also present in the fetal lamb and are capable of stimulating insulin release from the ovine fetal pancreas and of altering the insulin response to glucose. Glucose, however, is believed to the major energy substrate for both fetal and placental metabolism. The administration of glucose provoked a significant release of plasma insulin at the earliest time tested (d110) through until d145 of gestation. Peak insulin responses occurred at +20-30 min with insulin concentrations declining by the +30-60 min mark. This study design does not allow these responses to be subjected to rigorous kinetic analysis but it is possible to conclude that the insulin response is present and that the time course of the response is similar throughout the last 35 days of pregnancy in sheep fetuses. These observations agree with the results of Fowden (1980a) based on studies in fetuses between d124 and 140 of gestation and other studies performed by Phillips et al (1978). Our studies extend the time and duration of the insulinotrophic effect of glucose back to d110 of gestation. Shelley et al. (1973) showed that glucose provoked insulin release from perfused fetal pancreas as early as d50 of gestation. These results suggest good agreement between *in vitro* and *in vivo* observations and suggest that other factors such as hypoxia or surgical stress which induce catecholamine release (Shelley 1973), may have contributed to the apparent lack or sluggishness of the insulin response that has been reported previously.

Although the peak insulin response in fetuses tested between d125 and d130 of gestation was less than that in fetuses at d140-145, this was associated with smaller increases in peak glucose concentrations. There was no change in the summed insulin release over summed glucose concentration ratio. *In vitro* experiments demonstrated that insulin release per unit of glucose administered decreased between d50 to d100 of gestation, but did not change thereafter to term (Shelley et al. 1973). Our results are in

accord with these observations. Peak glucose and insulin concentrations in adult non-pregnant sheep were significantly greater ($p<0.05$) than those in the fetus. The rapid decline in glucose and insulin concentrations in the fetus following the bolus injection of glucose may be explained by the flux of glucose from the fetal into the maternal circulation. Hay et al. (l984b) have demonstrated that approximately 50% of radioactive glucose infused into the fetus is extracted by the placenta and that approximately one half of this reaches the maternal circulation.

In conclusion, the fetal insulin response to an acute glucose challenge is present by d110 of gestation, and the ratio of insulin released per unit glucose elevation did not change in fetal sheep between d110-145 of gestation, suggesting that it does not change or mature in fetal sheep over the last one third of gestation. In addition, these studies have also demonstrated that the insulin response, expressed as the ratio of insulin released per unit glucose elevation, is similar between fetal and adult sheep. This is of interest since it supports the suggestion that the fetal insulin response is not sluggish or immature but rather that the sensitivity of the endocrine pancreas is similar in fetal sheep between d110 and term, and in fetal and adult sheep.

Having established the insulin response to an glucose load is present in fetal sheep during the last one-third of gestation we then proceeded to investigate the fetal insulin response to GRP.

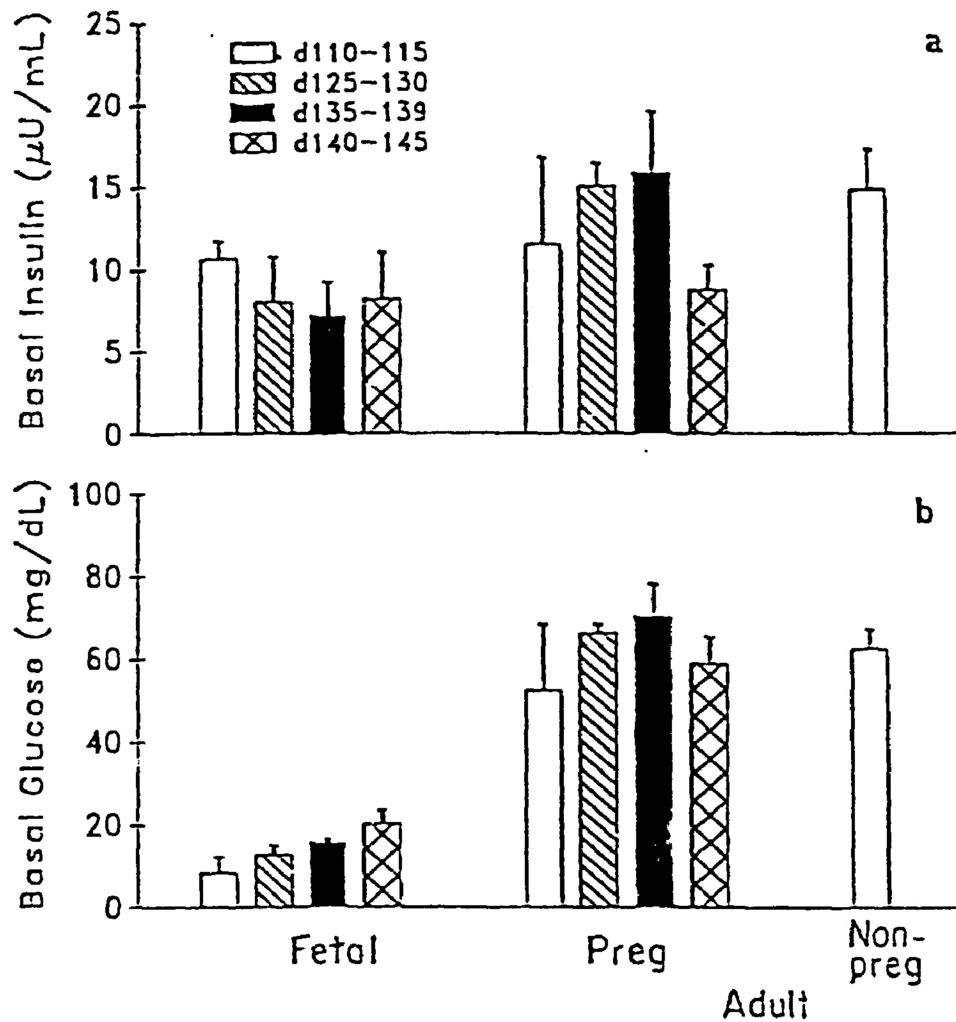

Figure 1. Bar graphs of mean ± SEM of (a) basal insulin and (b) basal glucose for fetal and adult pregnant (Preg) sheep measured between d110 and d145 of gestation and non pregnant (Non-preg) sheep. (d110-115; n=5, d125-130; n=6, d135-139; n=5, d140-145; n=3).

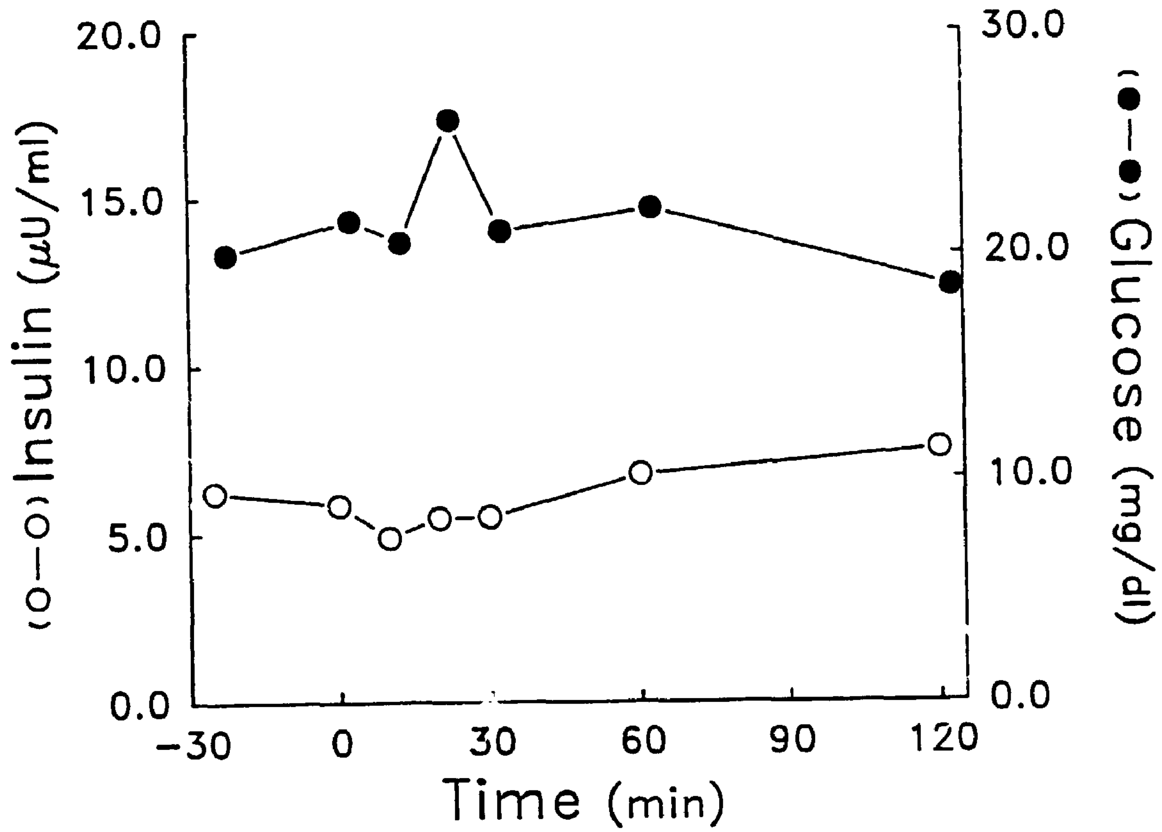

Figure 2. Mean of plasma glucose (●) and plasma insulin (○) concentrations before and after a bolus injection of 1.6 g/kg of L-glucose into fetal sheep (n=2) between d125-130 of gestation.

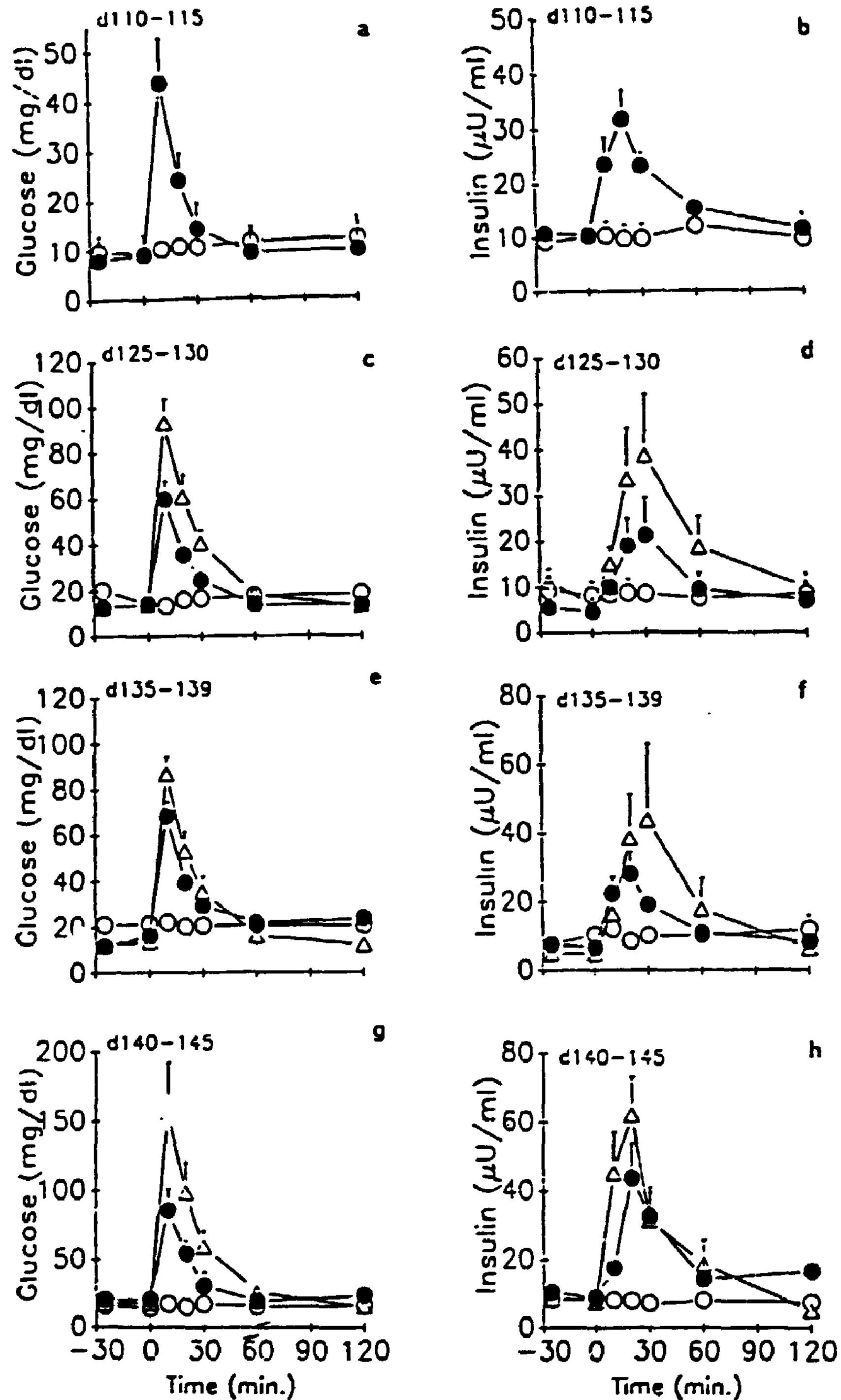

Figure 3. Mean ± SEM of plasma glucose (left) and plasma insulin (right) before and after a bolus injection of saline (○), 0.8g/kg glucose (●), and 1.6 g/kg glucose (△) in fetal sheep tested between d110-115; n=5 (a,b), d125-130; n=6 (c,d), d135-139; n=5 (e,f), and d140-145; n=3 (g,h). (Where no SEM is shown, error is less than the size of the data point).

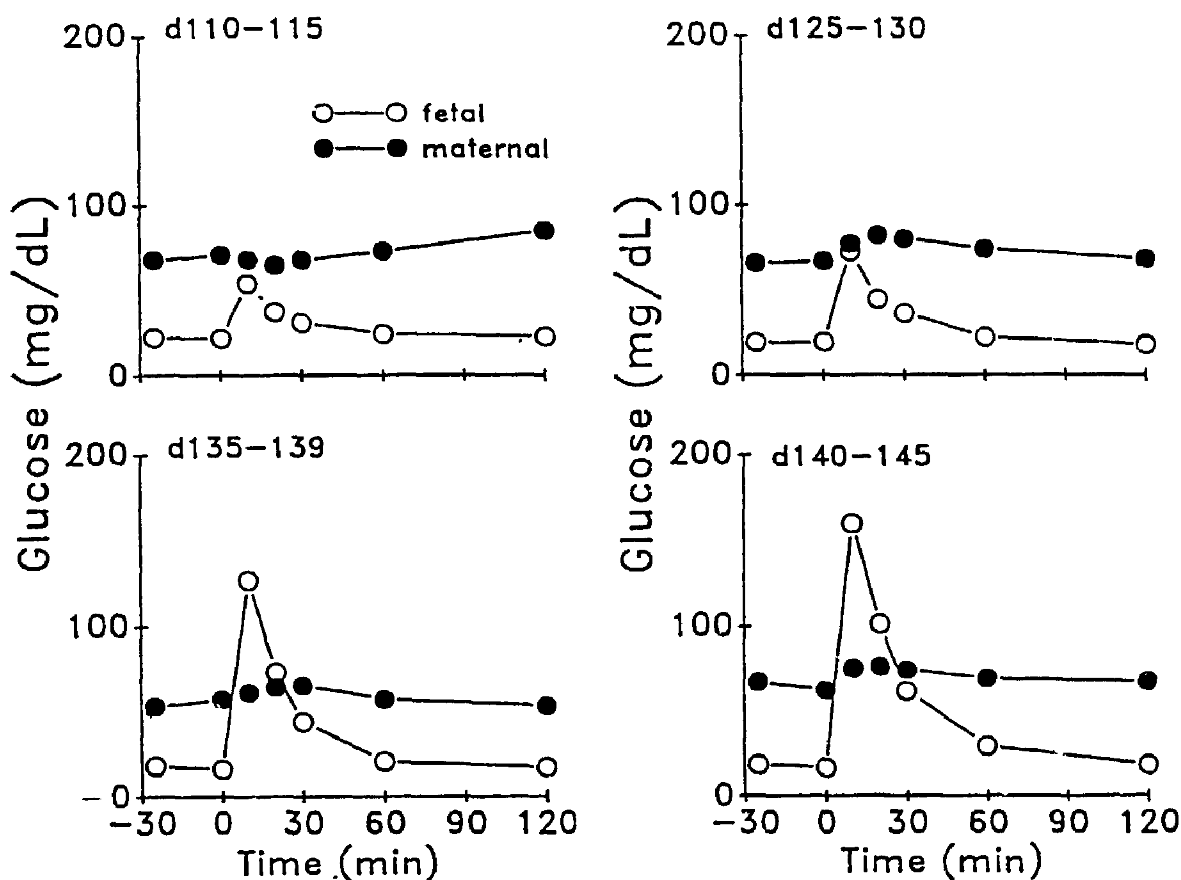

Figure 4. Plasma glucose concentrations measured in fetal (○) and maternal (●) plasma of a representative sheep given a bolus injection of glucose (0.8g glucose/kg at d110; 1.6g glucose/kg at all other ages) to the fetus at various gestational ages indicated.

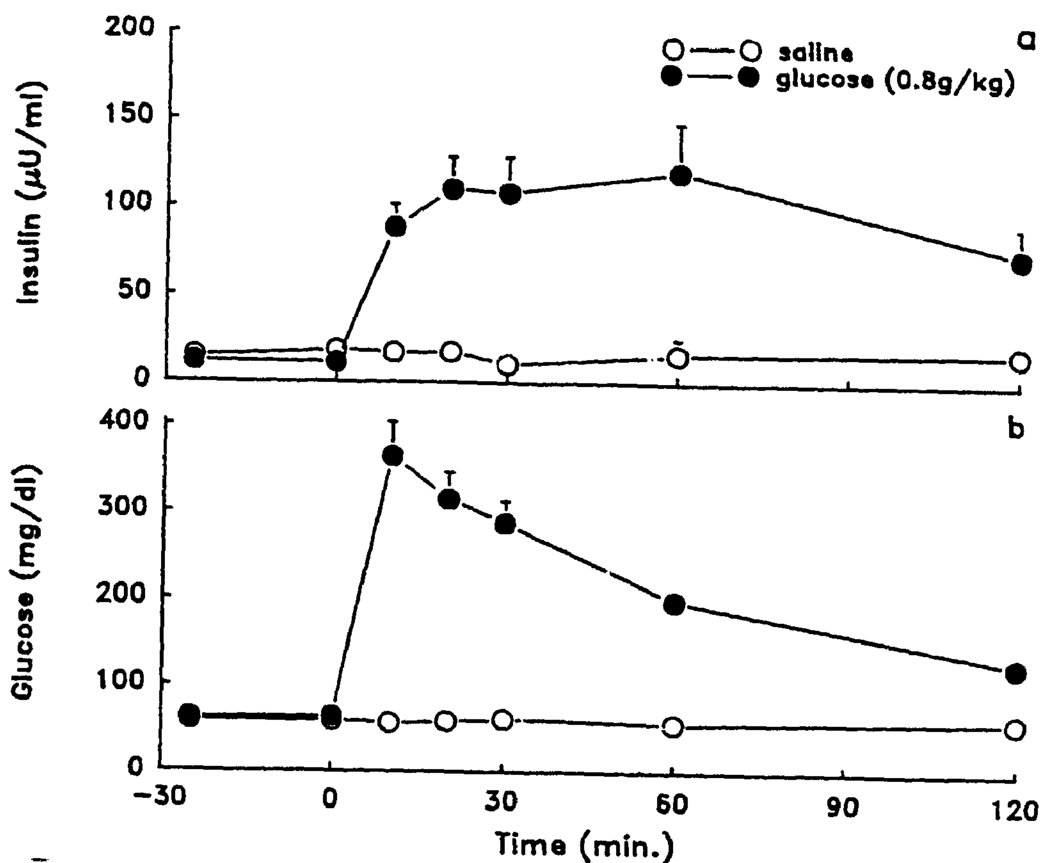

Figure 5. Mean ± SEM of insulin (a) and glucose (b) following bolus injection of saline (○); n=4, and glucose (●); n=5 into adult non pregnant sheep. (Where no SEM is shown, error is less than the size of the data point).

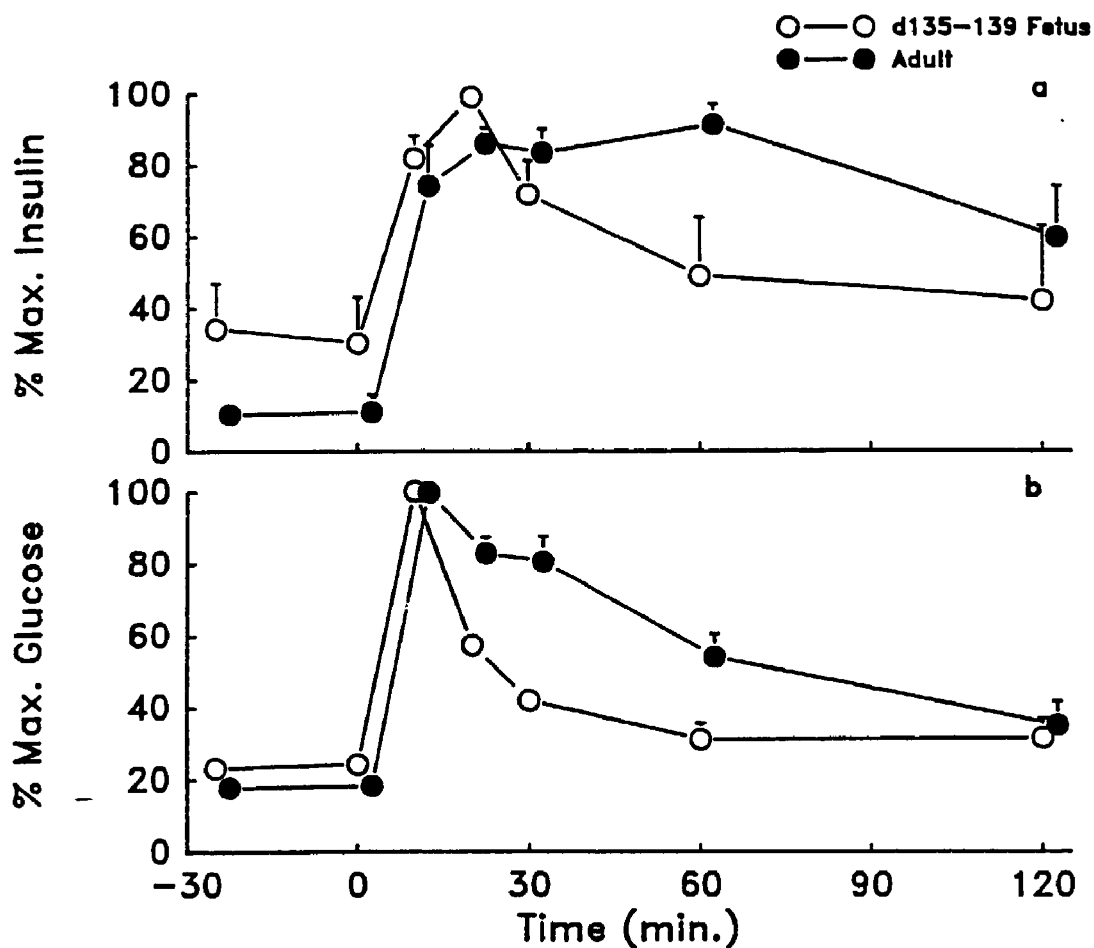

Figure 6. Mean ± SEM of insulin (a) and glucose (b) expressed as a percentage of maximum (max.) concentration reached for d135-139 fetal sheep (O); n=5, and adult non pregnant sheep (●); n=5. (Where no SEM is shown, error is less than the size of the data point).

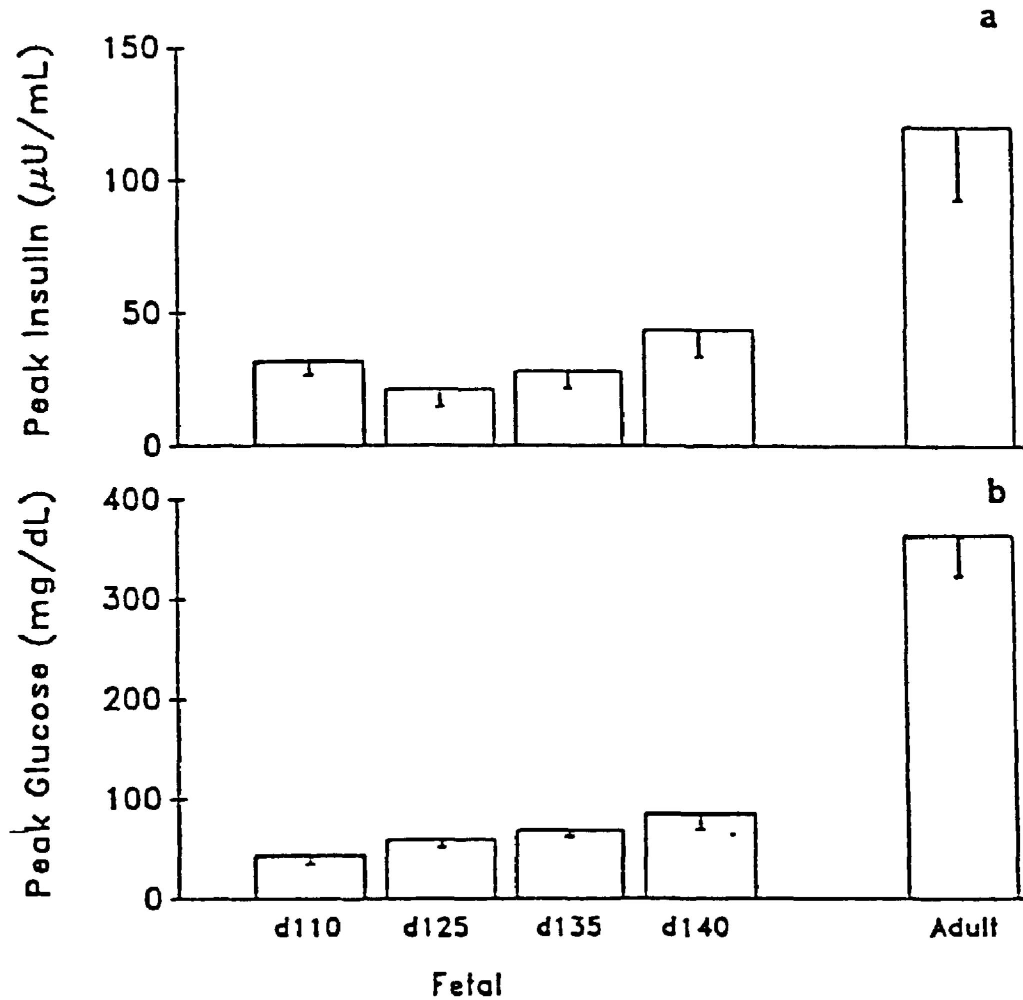

Figure 7. Mean ± SEM of peak insulin (a) and peak glucose concentrations (b) in fetal sheep between day 110 (d110) and term (d140) and in adult non pregnant sheep (Adult). (d110; n=5, d125; n=6, d135; n=5, d140; n=3, Adult; n=5).

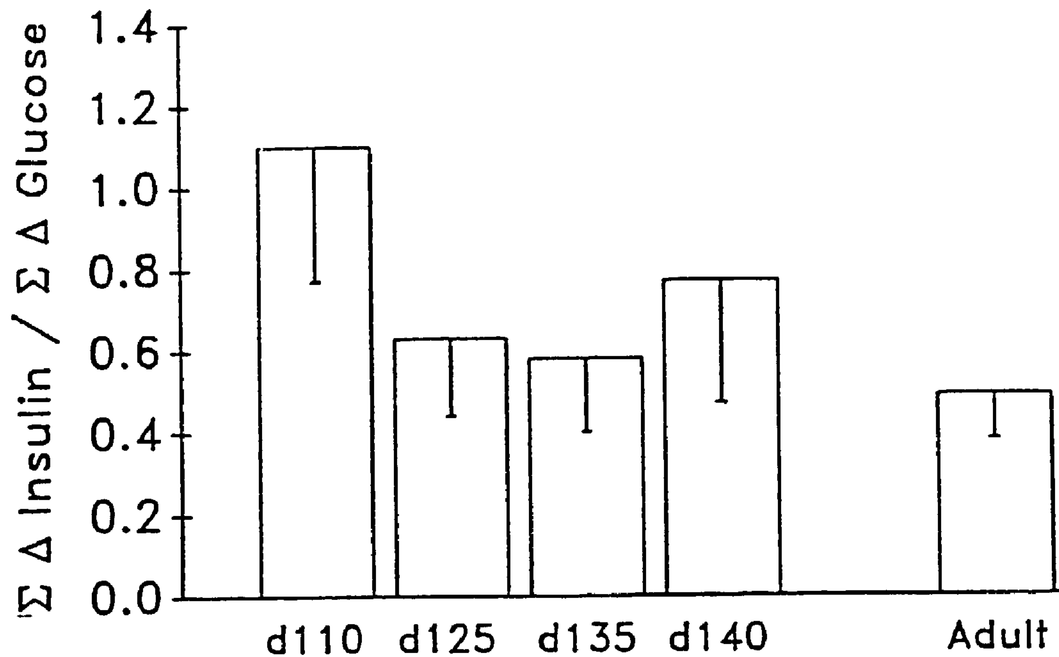

Figure 8. Mean ± SEM of the summed change in plasma insulin concentrations over the summed change in plasma glucose concentrations measured in fetal sheep between day 110 (d110) and term (d140) and in adult non pregnant sheep (Adult). (d110; n=5, d125; n=6, d135; n=5, d140; n=3, Adult; n=5)

5. PANCREATIC ENDOCRINE RESPONSE TO GASTRIN RELEASING PEPTIDE (GRP) ADMINISTRATION TO ADULT NON-PREGNANT SHEEP

5.1. INTRODUCTION

IV administration of the GRP has been shown to produce a wide spectrum of potent effects in several different monogastric species. On IV administration into conscious dogs, bombesin and GRP equipotently elevated systemic plasma levels of insulin, PP and pancreatic glucagon, (McDonald et al., 1983) and, in the isolated perfused canine pancreas (Ipp and Unger, 1979), bombesin stimulated the release of insulin and glucagon without affecting somatostatin release. Similarly, in man, IV infusions of bombesin (Ghatei et al., 1982) and GRP (Knigge et al., 1984) produced significant elevations in plasma insulin, PP and glucagon concentrations. However, in another study, it was reported that GRP infusion in man did not produce elevations of these pancreatic hormone levels but did enhance glucose stimulated insulin release (Wood et al., 1983). In an isolated perfused porcine pancreas preparation, GRP dose dependently stimulated the secretion of insulin and PP, inhibited somatostatin secretion, and did not affect pancreatic glucagon secretion (Knuhsten et al., 1987). GRP administration into mice (Pettersson and Ahren, 1977) resulted in significant elevations of plasma glucagon and insulin levels. Studies on the effects of GRP on the endocrine pancreas of the rat have produced equivocal and even apparently conflicting results. A number of technical factors may account for certain of the apparent discrepancies seen regarding the effects of GRP/bombesin on the rat endocrine pancreas. One factor of importance may be the use of differing doses of GRP, as a recent study in the anaesthetized rat model reported that low dose GRP administration resulted in inhibition of glucose-, meal-, and GIP- induced insulin release but that the inhibitory effect on glucose-stimulated insulin release was not seen at higher doses of GRP (Schnuerer et al., 1987).

There have been few studies on the effect of GRP in ruminants. Bloom et al (1983b) reported that, in the conscious calf, IV GRP administration elevated pancreatic glucagon and PP plasma levels and was a potent insulin secretagogue. This group have also reported that the stimulation of insulin release by GRP in conscious calves is dependent on circulating glucose and amino acid concentrations (Bloom et al., 1984). The effect of GRP administration on the ovine endocrine pancreas has not yet been examined.

The purpose of these experiments was to determine the responses of plasma glucose and plasma insulin and glucagon levels to IV administration of GRP into adult non-pregnant sheep. We administered GRP intravenously to adult non-pregnant sheep at the same doses of GRP employed in previous studies on other species (McDonald et al., 1981). We compared pancreatic endocrine reponses to GRP that we obtained in sheep to those performed previously in other species. Specifically we asked: (1) Does IV administration of GRP stimulate elevations in plasma insulin or glucagon concentrations in adult non-pregnant sheep? and (2) How does IV administration of GRP alter plasma glucose concentrations in adult non-pregnant sheep?

5.2. MATERIALS AND METHODS

5.2.1. Animals

Four adult non-pregnant sheep during seasonal anestrus had catheters surgically implanted into the femoral artery and vein as previously described (section 3.2). The sheep received post-operative care for seven days as described in section 3.3 and were provided with grain, hay, and fresh water daily at around 0800 hr and again around 1500 hr (see Appendix II for food composition). On the day prior to an experiment, animals were fed at 0800 hr but received only fresh water at 1500 hr. Food was withheld overnight and during the following day while the experiments were being carried out.

5.2.2. Experimental Protocol

All experiments were performed between 0800 hr and 1200 hr which was following fasting of the animals for approximately 17 to 21 hr. On separate days, a 30 min infusion of synthetic GRP (Bachem, Torrence, California) dissolved in saline just prior to administration, was given at the following doses: 0 (control saline infusion; SALINE), 25 pmol/kg.hr (GRP25), 100 pmol/kg.hr (GRP100), 300 pmol/kg.hr (GRP300), and 600 pmol/kg.hr (GRP600). The different doses of GRP were administered as a 30 min infusion via the femoral venous catheter. A volume of infusate equal to the catheter dead space volume (3.0 - 4.0 ml) previously determined at the time of surgery, was displaced into the catheter just prior to the onset of the infusion. Femoral arterial blood samples (4.0 ml) were collected -15 , -1, +5, +10, +20, +30, +35, +45, and +60 min relative to the onset of the infusion. The samples were processed and stored as described previously (section 3.4). Plasma insulin and glucagon concentrations were determined in all samples taken using RIA techniques as described previously (section 3.6). Plasma glucose concentrations were analyzed as described in section 3.5.

5.2.3. Data Analysis

Plasma glucose and hormone levels in response to graded doses of GRP are presented as the mean ± SEM of change over the mean of the two pre-injection values for each individual animal. The effect of time and treatment on plasma glucose, glucagon, and insulin concentrations was assessed using a two way analysis of variance. A value of $p<0.05$ was considered to represent a statistically significant difference.

5.3. RESULTS

During saline infusions there was no significant change in plasma glucose, insulin, or glucagon concentrations. In response to GRP administration, prompt elevations of plasma insulin levels occurred with peak values of 5 ± 3 μU/ml, 32 ± 6, and 40 ± 2 μU/ml attained at +5 to +10 min following the onset of the infusion of GRP100, GRP300, and GRP600, respectively (Fig. 9). Thereafter insulin levels fell towards basal values despite continued GRP infusion. Statistically significant ($p<0.05$) elevations of plasma insulin levels from baseline were observed in the sheep between +5 to +20 min during the infusion of GRP300 and GRP600 (Fig. 9). Administration of the lower doses of GRP (GRP25 and GRP100) did not produce any significant change in plasma insulin concentrations.

Plasma glucagon levels were significantly elevated at the GRP100, GRP300, and GRP600 but not GRP25 or GRP100 infusion doses (Fig. 10; $p<0.05$). Peak glucagon elevations of 60 ± 22, 104 ± 26, 164 ± 39 pg/ml occurred between +20 and +30 min relative to the onset of the infusion of GRP100, GRP300, and GRP600 respectively (Fig. 10). Following peak values, plasma glucagon concentrations gradually declined to base line by + 60 min (Fig. 10).

GRP administration to adult non-pregnant sheep produced dose dependent increases in circulating plasma glucose concentrations (Fig.11). Statistically significant elevations in plasma glucose levels above baseline ($p<0.05$) occurred at +20 to +35 min during the infusion of GRP600 (11 ± 3 mg/dl; Fig. 11). Plasma glucose concentrations were not significantly altered during or after the administration of GRP25, GRP100, or GRP300.

The time course of the changes in plasma insulin, glucagon, and glucose concentrations during the infusion of GRP600 to adult sheep are compared in Fig. 12.

Administration of GRP600 resulted in rapid transient elevations in plasma insulin concentrations with peak elevations occurring at + 5 min and more gradual increases in plasma glucagon and glucose concentrations with peak plasma glucagon (164 ± 39 pg/ml) and glucose (11 ± 4 mg/dL) concentrations occurring at +20 min.

5.4. DISCUSSION

From these studies it is apparent that GRP is a potent secretagogue of insulin and glucagon in adult non-pregnant sheep. This is consistent with results obtained from studies in other ruminant (Bloom et al., 1983b) and non-ruminant species (McDonald et al., 1981 and 1983; Kaneto et al., 1988; Pettersson and Ahren, 1987; Knuhsten et al., 1987) and suggests that GRP may be involved in the intricate control of pancreatic endocrine function in sheep.

With minor exceptions, the responses of the sheep to GRP infusions observed in this study were qualitatively similar to responses in dogs (McDonald et al., 1981). Although plasma glucagon and insulin levels were elevated following GRP administration in both sheep and dogs, the dose dependent increments in plasma glucose concentrations which occurred in the sheep were opposite to the dose dependenat decrements observed in the dog. These differences do not result from a direct effect of GRP on the liver (Bloom et al., 1984) but probably represent species differences in the responses of hepatic and peripheral tissues to elevations of plasma insulin and glucagon concentrations as outlined previously (section 1.2). In brief, in contrast to non-ruminants, ruminants are relatively insensitive to insulin-induced suppression of hepatic glucose production, and the effect of insulin on glucose movement into peripheral tissues may be slower in ruminants compared to non- ruminants (Brockman, 1978). In both ruminants and non-ruminants, glucagon is a potent promoter of hepatic glucose production (Brockman et al., 1975b). GRP induces

elevations in circulating concentrations of insulin and glucagon in both sheep and dogs. However, elevations in plasma glucagon levels probably has the predominant effect in sheep, increasing hepatic glucose production and thus producing the observed rise in plasma glucose. In the dog, insulin elevations induced by GRP is the predominant factor producing an initial decrease in hepatic glucose production which results in an initial fall in glucose levels. These species differences have been confirmed in glucose turnover studies performed during GRP administration to dogs (McDonald et al., 1981) and sheep (McDonald et al., 1988).

Little is known about the mechanism(s) of action by which GRP stimulates hormone release from the endocrine pancreas. Studies to date have been performed mostly in monogastric species. BLI has been detected in extracts of pancreas from human, pig, rat, and guinea pigs (Ghatei et al., 1984). Recent immunocytochemical studies report that GRP positive nerve cell bodies are present in intrapancreatic ganglia of the pig and that GRP positive nerve fibers penetrate the islets (Knuhtsen et al., 1987). Similarly, GRP positive nerve fibres are present at the periphery of and penetrating into pancreatic islets of dogs (A.J.M. Buchan and T.J.M. McDonald, unpublished results). These studies demonstrate that a close anatomical relationship between GRP-containing nerve fibers and islet cells exists and support the hypothesis that in monogastric species, GRP is released from local nerves where it may act directly to stimulate hormone secretion from endocrine pancreatic cells. This suggestion is supported by *in vitro* studies which have demonstrated that GRP is capable of stimulating insulin release from the isolated perfused pancreas preparation of pigs (Knuhtsen et al., 1987), dogs (Ipp and Unger, 1979), and rats (Martindale et al., 1982), from isolated islets (Alwmark et al., 1986), and from neoplastic β-cells in culture (Swope and Schonburn, 1984).

Information is also scarce as to the mechanism by which GRP is released from nerves. Electrical stimulation of the vagus nerve in the isolated perfused pancreas of pigs resulted in elevations of GRP and insulin levels in the pancreatic venous effluent (Knuhtsen et al., 1985). This release of GRP by parasympathetic nerve stimulation was abolished by hexamethonium but not atropine administration, suggesting that GRP is released from post-ganglionic vagal fibers which innervate the islets (Knuhtsen et al., 1985). Marked species differences seem to exist however since Bloom et al (1980) report that in the conscious calf, stimulation of the splanchnic nerve, but not the vagus nerve (Adrian et al., 1983), results in elevations of BLI in the systemic circulation. The release of GRP following splanchnic nerve stimulation was unaffected by post-ganglionic adrenergic or cholinergic blockade but was abolished by hexamethonium, suggesting that GRP was released from post-ganglionic sympathetic fibers. Unlike the study in the pig (Knuhtsen et al., 1985), the study in the calf (Bloom et al., 1980) measured systemic levels of BLI. Immunocytochemical studies have demonstrated that GRP is confined to post-ganglionic sympathetic nerve terminals in the GI tract of the calf (Bloom et al., 1983b). Bloom and co-workers (1984) have also reported that preliminary experiments suggest that feeding is associated with elevated plasma BLI. Collectively these findings suggest that, in ruminants, GRP may be released under physiological conditions from post-ganglionic sympathetic nerves into the systemic circulation where it may act directly on the β-cell to stimulate insulin release, or act indirectly by stimulation of the release of other insulinotrophic hormones. However, this suggestion remains to be confirmed.

In conclusion, GRP adminstration to adult non-pregnant sheep produced dose dependent elevations in plasma insulin and glucagon concentrations and these were associated with dose dependent increments in plasma glucose levels. These effects of GRP on the endocrine pancreas are similar to those reported previously in other species.

These results suggest that, as in other species, GRP also may be important in the intricate control of pancreatic endocrine function and hence metabolic homeostasis in adult sheep. Species differences exist however in the potency of the hormone releasing activity of GRP, and the neural release mechanisms for GRP. The reasons for these species differences are unclear.

Having shown that GRP stimulates insulin and glucagon release in adult sheep, the effects of GRP were next examined in fetal and pregnant sheep and compared to those observed in adult non-pregnant sheep.

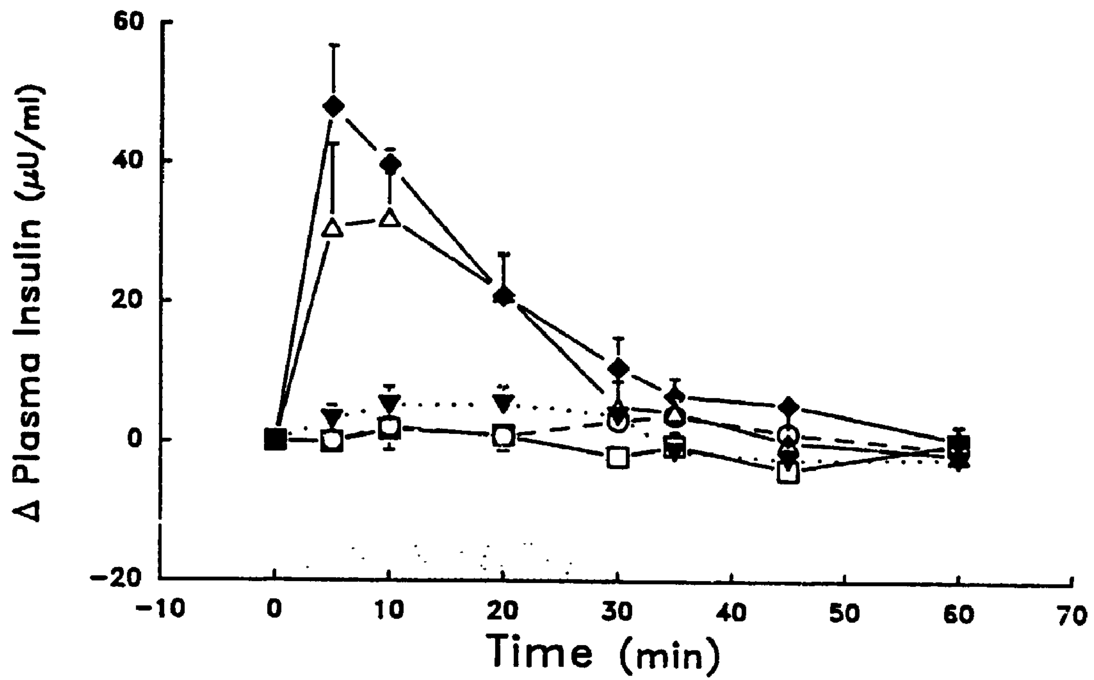

Figure 9. Mean ± SEM of the change over baseline of plasma insulin responses to 30-minute infusion of saline (□), 25 (○), 100 (▼), 300 (△), and 600 (◆) pmol/kg·hr of GRP in four adult non pregnant sheep following overnight fasting. (Where no SEM is shown, error is less than the size of the data point).

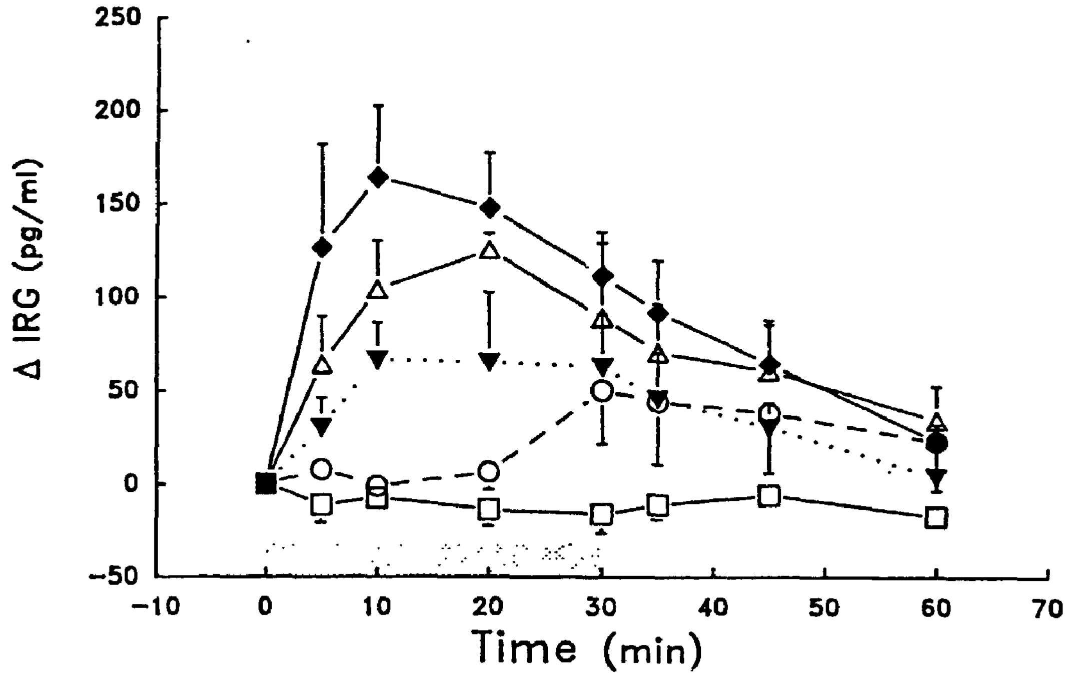

Figure 10. Mean ± SEM of the change over baseline of plasma glucagon (IRG) responses to 30 minute infusion of saline (□), 25 (○), 100 (▼), 300 (△), and 600 (◆) pmol/kg·hr of GRP in four adult non pregnant sheep following overnight fasting. (Where no SEM is shown, error is less than the size of the data point).

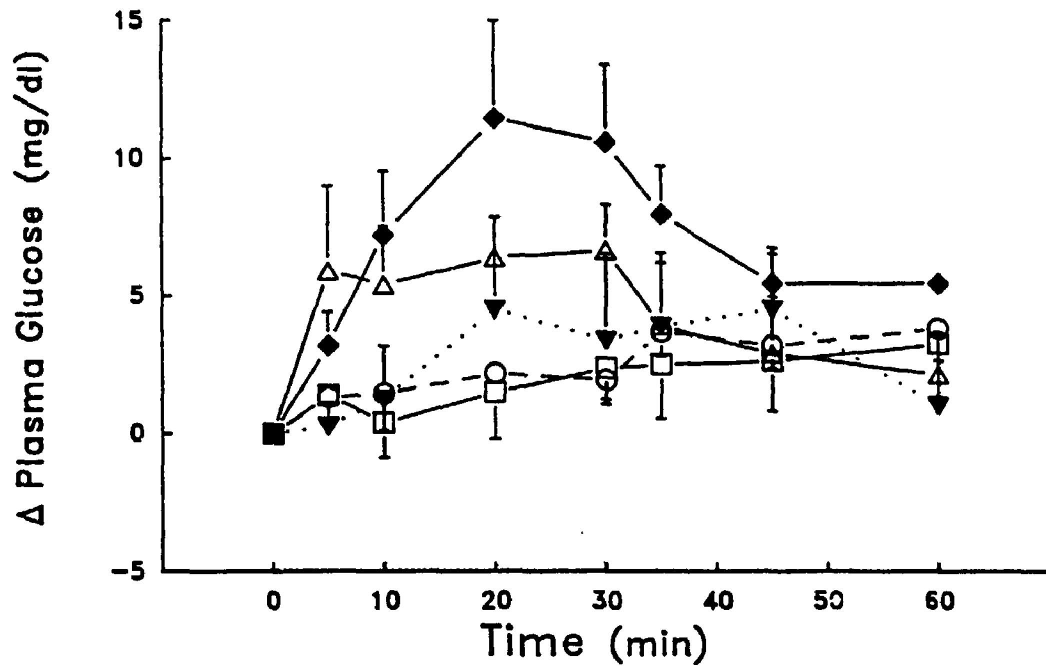

Figure 11. Mean ± SEM of the change over baseline of plasma glucose responses to 30-minute infusion of saline (□), 25 (○) ,100 (▼), 300 (△), and 600 (◆) pmol/kg·hr of GRP in four adult non pregnant sheep following overnight fasting. (Where no SEM is shown, error is less than the size of the data point).

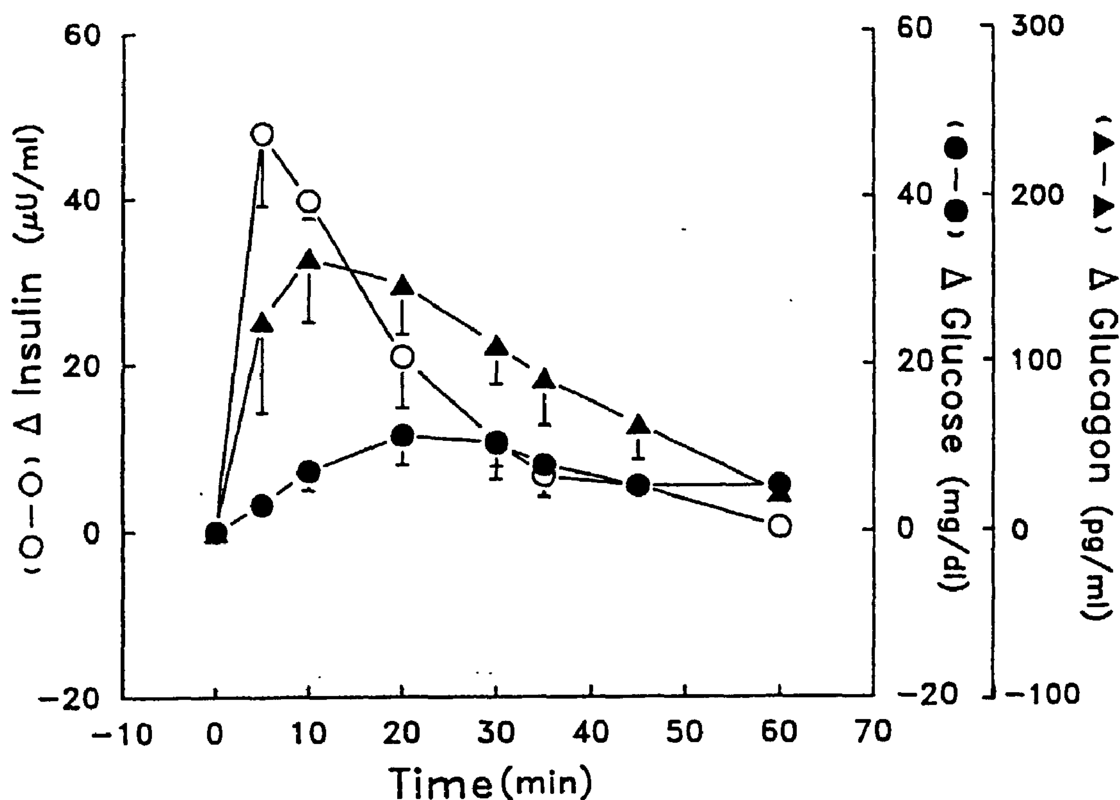

Figure 12. Mean ± SEM of the change over baseline of plasma glucose (●), insulin (○), and glucagon (▲) concentrations in four adult non pregnant sheep following a 30 minute intravenous infusion of 600 pmol/kg·hr of GRP.

6. PANCREATIC ENDOCRINE RESPONSE TO GRP IN FETAL AND PREGNANT SHEEP

6.1. INTRODUCTION

Many GI peptides, such as GRP, are capable of stimulating insulin release in the adult. The role GI peptides have in influencing fetal insulin concentrations has not been examined in the fetus of any species but is of great interest since insulin is important in determining proper fetal growth (Milner and Hill, 1984).

During pregnancy, the fetus receives glucose by transplacental transfer from the mother. It is believed that glucose is the major energy substrate to the fetus and that fetal glucose availability ultimately determines fetal growth (Fowden, 1985). Factors that influence glucose or insulin concentrations in the fetus directly, or indirectly through altering maternal glucose or insulin secretion, could also affect fetal growth and development. Carbohydrate metabolism during pregnancy undergoes massive changes as outlined previously (section 1.3). There are several factors which have been implicated as mediators of these changes (see section 1.3.2) and more recently these have included GI peptides. Gastrin and VIP (Attia et al., 1984) as well as galanin (McDonald, personal communication) are present in placenta extracts. Circulating levels of gastrin are significantly higher in pregnant compared to non-pregnant women and high VIP and gastrin concentrations are present in human cord blood (Attia et al., 1984). These results suggest that the placenta may contribute significantly to the circulating fetal and/or maternal plasma pools of VIP and gastrin. Whether the placenta is a source of other GI peptides, such as GRP, remains to be determined. The function of these GI peptides derived from the placenta during pregnancy is also unclear. A highly purified rat placenta extract has been shown to stimulate insulin release in adult rats, and from isolated neonatal rat islets (Sodoyez-Goffaux, et al., 1981). However, the identity of the insulinotrophic agent present in this extract is not known.

Other studies performed in pregnant women report that basal plasma concentrations of, and meal-stimulated release of, motilin, VIP, somatostatin, PP and GIP are reduced during pregnancy (Jenssen et al., 1988). In addition, the ability of these peptides to influence insulin secretion is diminished in pregnant compared to non-pregnant women (Lorrain et al., 1977; Hornnes et al., 1978). The results obtained in these experiments suggest that alterations in circulating insulin and glucose concentrations and the changes in metabolism which occur in women during pregnancy may be mediated, at least in part, by the attenuated release of, or reduced action of, one or more of the GI peptides. Determining factors which are responsible for changes in carbohydrate metabolism during pregnancy is of interest since pathological changes resulting in gestational diabetes are not uncommon in pregnant women. Furthermore, any effect of GI peptides on the maternal endocrine pancreas will in turn affect the fetus.

The purpose of the next series of experiments was to examine the hypothesis that GRP modulates insulin concentrations in the fetal sheep. This effect may occur due to the action of GRP directly within the fetal compartment or GRP may influence fetal insulin levels indirectly by inducing changes in plasma insulin and glucose concentrations in the maternal compartment. This in turn would alter plasma glucose and insulin levels in the fetus and hence fetal growth.

The following specific questions were addressed: (1) Does GRP administration stimulate insulin release in fetal sheep over the last one-third of gestation? (2) Does the insulin response to GRP in fetal sheep change over the last one-third of gestation? (3) How does the insulin response to GRP in adult pregnant sheep compare to that observed in adult non-pregnant sheep? (4) Does GRP administration to pregnant sheep alter fetal plasma glucose or insulin concentrations?

6.2. MATERIALS AND METHODS

6.2.1. Animals

Eleven time-mated pregnant sheep and five non-pregnant sheep were used in this portion of the study. Catheters were placed into the fetal carotid artery, fetal jugular vein, and amniotic fluid cavity using surgical techniques described in section 3.2. A maternal femoral artery and vein were also catheterized at this time. Surgery was performed on pregnant ewes and their fetuses between d100-105 of gestation and post-operative care was carried out for 7 days as described in section 3.3.

Under general anaesthesia, five adult non-pregnant sheep had catheters implanted into a femoral artery and vein. In addition, the ovarian blood vessels were ligated bilaterally and both ovaries were removed through a small abdominal incision. Post-operative care was carried out as described in section 3.3.

Animals received food and water twice daily (at around 0800 hr and again at around 1500 hr) as described in section 3.3 (see appendix II for food composition). Although the sheep consumed most of the food within the first 1 - 2 hr of receiving it, we have termed this feeding regime "fed *ad libitum*". In this series of experiments the animals continued to be fed *ad libitum* on days when GRP stimulation tests were performed. On these days, animals were fed at 0800 hr and again upon completion of the experiments on all the sheep in a given housing room (around 1600 hr).

6.2.2. Experiments in Non-pregnant Ovariectomized Sheep

Experiments were performed on five adult non-pregnant sheep fed *ad libitum* in which both ovaries were removed at the time of surgery. Sufficient amounts of synthetic porcine GRP (Bachem, Torrence, Ca.) necessary to acquire the desired doses was dissolved in 65 ml of sterile isotonic saline just prior to administration. A volume of infusate equal to the catheter dead space volume (determined at the time of surgery) was

infused into the catheter just prior to the onset of the infusion. GRP was administered via the femoral venous catheter at a rate of 2 ml/min for 30 min. at the following doses; 0 (control saline infusion, SALINE), 100 pmol/kg.hr (GRP100), and 600 pmol/kg.hr (GRP600) of GRP. Test substances were administered in a randomized order, on successive days, between 1200 hr and 1600 hr. Blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +10, +20, +30, +35, +45, and +60 min. relative to the onset of the infusion. Plasma glucose, insulin, and glucagon concentrations were determined as described (section 3.5 & 3.6).

6.2.3. Experiments In Pregnant Sheep

Similar experiments to those described above in non-pregnant sheep (section 6.2.2), were also performed in pregnant ewes fed *ad libitum* during the two periods (d117-128, n=4; and d133-141; n=4) between the studies conducted on their fetuses (see next section). The same doses of GRP per kg body weight (SALINE, GRP100, and GRP600) were prepared and administered as a 30 min. infusion via the maternal femoral venous catheter. Maternal arterial plasma samples were collected at -15,-1 min. before and +5, +10, +20, +30, +35, +45 and +60 min. after the onset of the infusion. Experiments were performed on separate days, in a randomized order between 1200 and 1600 hr and were carried out in certain pregnant ewes at more than one gestational age. In three pregnant sheep between d133-141 of gestation, both fetal and maternal arterial blood samples were collected at all the times indicated above. Fetal and maternal plasma glucose and insulin concentrations and maternal plasma glucagon concentrations were determined as described (section 3.5).

6.2.4. Experiments In Fetal Sheep

Experiments were performed in fetal sheep at three times during late gestation; between d110-117, n=4; d123-133, n=11; and d137-145, n=5. At each of these three

times in gestation, synthetic GRP (Bachem, Torrence, Ca.), prepared in 20 ml of saline just prior to the experiment as described, was infused at a rate of 0.2 ml/min for 30 min. into the femoral venous catheter at the following doses: 0 pmol/kg.hr (control saline infusion; SALINE), 100 pmol/kg.hr (GRP100), 600 pmol/kg hr (GRP600). These doses of GRP were based on estimated fetal body weight of 1.5 kg at d110-117, 2.25 kg at d123-133, and 3.5 kg at d137-d145 of gestation (Barcroft, 1945). Test substances were administered in a randomized order on successive days between 1200 hr and 1600 hr. Experiments were repeated in certain fetuses at more than one gestational age. Blood samples were collected and processed as described (section 3.4) from both the fetal and maternal femoral artery at 15 and 1 min. before starting the infusion, then at +5, +15, +30, +45 min. after the onset of the infusion into the fetus. Fetal and maternal plasma glucose and insulin concentrations and maternal plasma glucagon concentrations were determined using techniques described (section 3.5 & 3.6).

6.2.5. Data Analysis

Results are presented as the mean ± SEM of the change over the mean of the two pre-injection values for the number of animals indicated (n). The effect of time and treatment on glucose, glucagon, and insulin concentrations in plasma was assessed using a nested analysis of variance where numbers in treatment groups were equal, and using a two way unbalanced analysis of variance where there were unequal numbers between treatment groups. Multiple comparisons were then performed using Duncan Multiple Range test.

The sum changes in plasma insulin concentrations in fetal and adult sheep during GRP administration were calculated as the mean ± SEM of the summed increases over the average of the two pre-injection values for each individual animal. One way analysis of variance was used to test for significant differences in the sum change in plasma insulin

concentrations between treatment groups at each gestational age and between fetal, pregnant and non-pregnant sheep. Basal glucose, insulin, and glucagon concentrations were determined using the mean of the two pre-injection values for each of the individual animals in the groups indicated and were compared statistically using a one way analysis of variance. Differences were considered statistically significantly at $p<0.05$.

6.3. RESULTS

6.3.1. Basal Glucose, Insulin, and Glucagon Concentrations

Basal insulin concentrations measured in pregnant ewes increased significantly with gestational age to concentrations at d133-141 of gestation which were significantly greater than those measured in adult non-pregnant sheep (refer to Table 1; $p<0.05$). Maternal basal glucose concentrations measured in d133-141 pregnant ewes were significantly greater than those in non-pregnant sheep but were not different from basal glucose concentrations measured in pregnant sheep earlier in gestation (between d117-128). There was no significant difference between basal plasma glucagon concentrations measured in pregnant at different gestational ages or between d133-141 pregnant sheep and non-pregnant sheep.

Basal plasma insulin and glucose concentrations were significantly less in fetal sheep compared to those measured in adult pregnant sheep and non-pregnant sheep ($p<0.05$; refer to Table 1). In this study, basal insulin concentrations measured in the plasma of fetal sheep did not change significantly during the last one-third of gestation (between d110-d145) whereas fetal basal glucose concentrations were significantly higher in d110-117 fetal sheep compared to glucose concentrations measured later in gestation ($p<0.05$; refer to Table 1).

6.3.2. Experiments in Non-pregnant Ovariectomized Sheep

GRP600 administration to non-pregnant ovariectomized sheep fed *ad libitum* resulted in large rapid elevations in plasma insulin concentrations which were significantly greater than insulin levels following saline administration at + 5 to + 20 min. ($p<0.05$; Fig. 13). Peak plasma insulin elevations of 42 ± 8 μU/ml occurred at + 5 min. and then plasma insulin levels subsequently declined despite continued GRP infusion. Plasma pancreatic glucagon concentrations increased to 61 ± 18 pg/ml at + 20 min. and remained significantly elevated above baseline until the completion of the 30 min. infusion of GRP600 ($p<0.05$; Fig 13). Plasma glucose levels were significantly increased by approximately 10mg/dl over baseline during infusion of GRP600 ($p<0.05$; Fig. 13).

Infusion of the lower dose of GRP (GRP100) into the same animals produced a significant elevation in plasma insulin concentrations at + 5 min. (8 ± 4 μU.ml; $p<0.05$) but did not produce a statistically significant change in plasma glucose or plasma glucagon concentrations (Fig. 13).

6.3.3. Experiments in Pregnant Ewes

a) Responses to GRP Infusion in Pregnant Ewes Fed *ad libitum*

Administration of GRP600 into fed pregnant ewes between d133-141 of gestation resulted in significant, elevations in plasma insulin concentrations between +5 and +20 min. (Fig. 14; $p<0.05$). Peak plasma insulin elevations (32 ± 8 μU/ml) occurred within 5 min. after the onset of the GRP infusion but then declined despite continued GRP administration. Plasma glucagon levels were increased over baseline during the 30 min. GRP600 infusion into pregnant ewes fed *ad libitum*; statistically significant elevations over baseline in plasma glucagon occurred at +10 min. after the onset of the infusion of GRP (40 ± 3 pg/ml; Fig. 14; $p<0.05$). Elevations in plasma glucagon occurred more gradually than did insulin responses but were maintained throughout the GRP infusion. In pregnant

ewes fed *ad libitum*, there was no significant change in plasma glucose concentrations as a result of the administration of GRP (Fig. 14).

Infusion of GRP100 into d133-141 pregnant ewes fed *ad libitum* did not significantly affect plasma insulin, glucagon, or glucose concentrations when compared to pre-injection values or saline treated controls (Fig. 14).

b) Changes in Fetal Plasma Insulin and Glucose Concentrations During Maternal Infusion

Administration of GRP600 to the pregnant ewe did not result in any obvious change in either insulin or glucose concentrations measured in the fetal plasma (Fig. 15).

c) Effect of Gestational Age

The insulin response, expressed as the sum of the change over baseline of plasma insulin concentrations, to the infusion of GRP600 was not significantly different between pregnant sheep tested between d117-128 compared to those tested between d133-141 of gestation (Fig. 16). The magnitude of the elevations in plasma glucagon and glucose concentrations in response to GRP administration was also not significantly different between d117-128 and d133-141 of gestation (data not shown). As a result, responses obtained for pregnant sheep between d117-128 and d133-141 of gestation were pooled when presented in subsequent sections of this thesis.

6.3.4. Comparisons Between Pregnant and Non-pregnant Sheep

The insulin response, expressed as the sum of the change over baseline of plasma insulin concentrations, to the infusion of GRP600 was significantly greater in non-pregnant compared to pregnant sheep (Fig. 16; $p<0.05$). In both non-pregnant and pregnant sheep peak insulin elevations occurred within 5 min. after the onset of the infusion of GRP600, whereafter plasma insulin concentrations declined to values not statistically different from baseline by 30 min. in both pregnant and non-pregnant sheep (Fig. 17).

Administration of either dose of GRP into pregnant sheep did not produce a significant change in plasma glucose concentrations but in non-pregnant sheep, GRP600 administration caused a small but significant increase in circulating glucose levels ($p<0.05$). However, the sum change in plasma glucose and glucagon concentrations during the infusion of either dose of GRP was not significantly different between pregnant and non-pregnant sheep (data not shown).

6.3.5. Experiments in Fetal Sheep

The administration of either dose of GRP to d110-117 fetal sheep did not produce a statistically significant change in fetal plasma insulin or glucose concentrations (Fig. 18a). GRP100 administration to one fetus tested at d112 of gestation resulted in a very large elevation in fetal plasma insulin concentrations. As a result, the variations around the mean plasma insulin concentrations for d110-117 fetal sheep are substantial (refer to Fig. 18a. In fetuses tested at two times later on in gestation (d121-127 and d137-145), GRP administration at either dose continued to be without effect (Fig. 18 b&c). Maternal glucose and insulin concentrations were also not significantly altered as a result of the administration of either dose of GRP into the fetus (Fig. 19).

6.4. DISCUSSION

Results from this section demonstrate that GRP administration into pregnant sheep, resulted in significant, transient, elevations in plasma insulin concentrations, more gradual increases in plasma glucagon concentrations, and no significant change in plasma glucose concentrations. However, elevations in plasma insulin concentrations in response to a similar dose of GRP were significantly lower in pregnant compared to non-pregnant sheep. Furthermore, GRP administration to fetal sheep at doses which stimulated insulin release when administered to adult pregnant and non-pregnant sheep, did not produce

a detectable change in fetal plasma insulin concentrations. From these experiments it appears unlikely that GRP influences plasma insulin and glucose concentrations in the fetus by acting directly within the fetal compartment. Rather, it is more probable that GRP may act on the fetus indirectly by altering maternal plasma glucose and insulin concentrations which in turn influence fetal glucose and hence insulin concentrations by transplacental glucose transfer.

All experiments were conducted after a recovery period from surgery of at least 7 days. This allowed plasma glucose and insulin concentrations to stabilize and minimized the effects of anaesthesia and surgery. Experiments were also conducted at the same time of day and at similar times after feeding in order to minimize any effects of diurnal rhythms of the endocrine environment on the parameters that we have measured. Basal plasma glucose concentrations in fetal, adult pregnant, and non-pregnant sheep obtained in the present study, were relatively constant and were in the range of normal values for basal glucose that have been reported previously (Shelley, 1973). This suggests that the animals were in a metabolic steady state.

Basal insulin concentrations in pregnant ewes increased significantly with gestational age to concentrations which were significantly greater than those measured in non-pregnant sheep. Similar trends were noted in data presented previously in this thesis (see Chapter 4), and in studies performed by other investigators in sheep (Shelley, 1973) and in humans (Bleicher et al., 1964). Basal glucose concentrations measured in pregnant ewes were not significantly different between d117-128 and d133-141 of gestation but were significantly greater than those measured in non-pregnant sheep. These results are not consistent with studies performed by other investigators (Shelley, 1973; Bleicher et al., 1964) nor are they consistent with previous studies performed in this thesis (Chapter 4) which report that basal glucose concentrations increase with gestational

age. The lack of change in maternal basal glucose concentrations between d117-141 of gestation reported in the present study may be explained by the relatively small range of gestational ages that was examined. Fetal basal insulin concentrations were significantly lower than those in non-pregnant and pregnant sheep and did not change with gestational age. Similar trends were reported previously in this thesis in d135-d139 fetal sheep (Chapter 4). In the previous study reported in Chapter 4, basal insulin concentrations in d110 - 115 fetal sheep were statistically greater than those in later gestational fetuses and were not statistically different from basal insulin concentrations in adult sheep. Furthermore, basal glucose concentrations in the same group of fetal sheep (d110-115) in the previous study were significantly less than those reported in fetuses of a similar gestational age (d110-117) in this study. This difference in basal glucose concentrations measured in these fetal sheep between d110-115 of gestation probably also explains our inability to confirm our previous reports (Chapter 4) of increasing fetal basal glucose concentrations over the last one-third of gestation. Although these results are statistically significant they represent small differences in low values of basal glucose and insulin concentrations which are very close to the limits of assay sensitivity and accuracy. Thus, these inconsistent results which occurred between studies in this thesis, may be explained simply by inter-animal or inter-assay variations which inevitably occur. Other studies examining changes in basal glucose concentrations in fetal sheep with respect to gestational age have also yielded variable results. Certain investigators report that fetal basal glucose concentrations do not change with gestational age (Shelley, 1973) while others report that glucose concentrations are lower in fetuses later on in gestation (Davis, 1970).

IV administration of both doses of GRP produced significant increases in plasma insulin, glucagon, and glucose concentrations in non-pregnant ovariectomized sheep which were fed *ad libitum*. These responses are consistent with similar studies performed in conscious calves (Bloom et al., 1983b), and are analogous with previous studies presented in this thesis (see chapter 5) performed in intact, fasted, non-pregnant sheep studied during seasonal anestrus following overnight fasting. Similar responses would be expected in ovariectomized sheep and in intact sheep studied during seasonal anestrus since ovarian function is dormant at this time. Differences between fed and fasted sheep will be addressed in detail in the next chapter.

GRP administration at doses which stimulated insulin release in adult sheep, was ineffective at altering fetal plasma insulin and glucose concentrations in ovine fetuses tested over the last one-third of gestation. GRP100 administration to one d112 fetus did produce very large elevations in plasma insulin concentrations. However, since this response was not observed in similar experiments performed at any other gestational age tested we dismissed this result as an extraneous result. We therefore feel that GRP administration does not influence insulin release in fetal sheep. The inability to demonstrate an insulinotrophic effect of GRP in fetal sheep at the doses administered may be due to one of the following possibilities. The mechanism by which GRP stimulates insulin release from the pancreas may be functionally immature or GRP receptors may not be present in the fetus and may develop post-natally. This possibility is supported by experiments performed by Bloom and Edwards (1981) which have demonstrated that neurally mediated release of different pancreatic hormones becomes functionally effective at different stages of development of conscious calves. Specifically, neurally stimulated PP release is fully developed at birth, sympathetically stimulated release of glucagon develops between 24 hr to 3-4 wk of age but neurally mediated release of insulin remains

functionally immature even in 3-4 wk old calves (Bloom and Edwards, 1981). It is known that insulin is present in the ovine fetal endocrine pancreas early in gestation and that pancreatic insulin content increase rapidly from day 100 of gestation until term (Wiles et al., 1969). Further, in a previous section (chapter 5) we have demonstrated the mechanisms governing glucose-stimulated insulin release are present in the ovine fetus relatively early in gestation. Therefore it is possible that the mechanism by which GRP stimulates insulin release is different than that following glucose stimulation and that this mechanism develops at a later age. Whether there is a lack of GRP receptors or a post-receptor deficit in the fetus, remains to be determined. It is also possible that administration of the same dose of GRP, based on body weight, to both fetal and adult sheep does not produce similar elevations in circulating GRP concentrations. This may occur due to altered production and/or metabolism of GRP during fetal life. The placenta is known to possess extensive metabolizing capabilities (Challis and Olson, 1988) and the metabolic clearance rate (MCR) of another GI neuropeptide (VIP) is greater in the fetus than pregnant ewe (Shulkes et al., 1987). It is possible that the MCR of GRP is greater during fetal life. We have investigated this possibility in a subsequent section (Chapter 9). A further possibility is that administration GRP directly into the fetal circulation produces GRP concentrations perfusing the fetal pancreas which are unphysiological. Tissue and plasma concentrations of many other GI peptides are higher in the fetus than in the adult (Shulkes and Hardy, 1982). Endogenous levels of GRP in fetuses of any species has not been examined and will therefore be addressed in subsequent experiments (Chapter 9).

Although GRP administration stimulated insulin and glucagon release in both pregnant and non-pregnant sheep, the magnitude of their release was much less in pregnant compared to non-pregnant sheep. In addition, plasma glucose concentrations

were not observed to change during infusion of GRP into pregnant ewes. This is in contrast to the dose dependent increases in plasma glucose concentrations which resulted from GRP administration to non-pregnant sheep. Pregnancy is associated with marked changes in many physiological systems including cardiovascular, renal, and endocrine systems. In particular, the synthesis and metabolism of many different hormones, such as progesterone and estrogen, are altered significantly during pregnancy and other hormones, such as placental lactogen, are produced in vast quantities only at this time (Jones, 1989). Since differences between pregnant and non-pregnant animals are so numerous, it is difficult to discern which factor(s) are involved in mediating differences observed during pregnancy. The attenuated insulin response to GRP which occurred in pregnant compared to non-pregnant sheep may simply be due to the fact that similar doses of GRP administered to both pregnant and non-pregnant sheep resulted in lower circulating GRP concentrations in pregnant animals. This may occur in pregnant animals due to dilution of GRP within the 30% greater blood volume which is recognized to exist during pregnancy (Freinkel, 1980). Alternatively, the metabolic clearance rate of GRP may be accelerated in pregnant animals. This possibility will be addressed in a later study (Chapter 9).

It is also recognized that pregnancy is associated with numerous metabolic changes including a state of accelerated starvation (Freinkel, 1980), increased β cell mass, and increased sensitivity to glucose stimulation as well as a progressive development of peripheral insulin resistance (Baird, 1986). β-cell hypertrophy and hypersecretion does not compensate sufficiently to offset the developing peripheral insulin resistance which occurs during pregnancy and as a result glucose tolerance is impaired and this becomes more manifest as pregnancy advances. Impaired glucose tolerance during pregnancy may be due to the lack or reduction in the action of potentiators of glucose-stimulated insulin release, such as GIP in monogastric animals (Hornnes et al.,

1978) and GRP in ruminants (Chapter 6). This suggestion is supported in the fact that the ability of other GI peptides, such as GIP, to augment glucose-stimulated insulin release is attenuated in human pregnancy (Lorrain et al., 1977; Hornnes et al., 1978). GRP stimulates insulin release in a glucose dependent manner in rats (Greeley and Thompson, 1984; Martindale et al., 1982), man (Wood et al., 1983), and conscious calves (Bloom et al., 1984). Whether or not the ability of GRP to stimulate insulin release is glucose dependent in sheep, remains to be determined. Further, whether the ability of GRP to potentiate glucose-stimulated insulin release is attenuated in pregnant compared to non-pregnant sheep is also unknown. These questions will be addressed in the following section (Chapter 7).

The placenta is well recognized as an important endocrine gland which is responsible for an extensive change in the endocrine environment of pregnancy. Numerous different peptides and steroid hormones have been identified within the placenta and the list is still rapidly increasing (Attai et al., 1984; Jones, 1989). Endocrir factors which are elevated during pregnancy and can induce pregnancy associateu changes in carbohydrate metabolism which occur are reviewed in section 1.3.2. They include glucocorticoids, prolactin (PRL), placental lactogen (PL), and estrogen (E_2) and (P_4). There is strong evidence supporting the role for E_2 and P_4 in producing an impaired glucose tolerance (Bender and Chickering, 1985; Baird, 1986) as well as other characteristic metabolic changes of pregnancy (Aerts et al., 1980). The involvement of glucocorticoids, PRL, and PL is less clear. Whether or not one or more of these factors may be responsible for alterations in the ability for GRP to stimulate insulin release in pregnant sheep is not known and will be addressed in a following section (Chapter 8).

Pregnancy is associated with simultaneous changes in many different factors. In order to examine factors mediating pregnancy associated changes, it is necessary to development an experimental model which permits manipulation of individual factors while maintaining all other variables relatively constant. We have attempted to discern which factor(s) is/are responsible for the attenuated effect of GRP during pregnancy in sheep. These studies are described in Chapter 8.

	Glucose (mg/dl)	Insulin (μU/ml)	Glucagon (pg/ml)
FETAL SHEEP			
d110–117	17 ± 1	6 ± 1	
d123–133	14 ± 1	6 ± 1	
d137–145	15 ± 1	4 ± 1	
PREGNANT SHEEP			
d117–128	72 ± 5	14 ± 2	62 ± 13
d133–141	72 ± 3	22 ± 2	95 ± 17
NON–PREGNANT SHEEP			
	60 ± 1	12 ± 1	112 ± 8

Table 1. Basal plasma glucose, insulin, and glucagon concentrations calculated as the mean ± SEM of the two pre-injection values for each of the individual fetal sheep between d110-117; n=4, d123-133; n=11, and d137-145; n=5, pregnant sheep between d117-128; n=5, and d133-141; n=5, and non pregnant sheep; n=5.

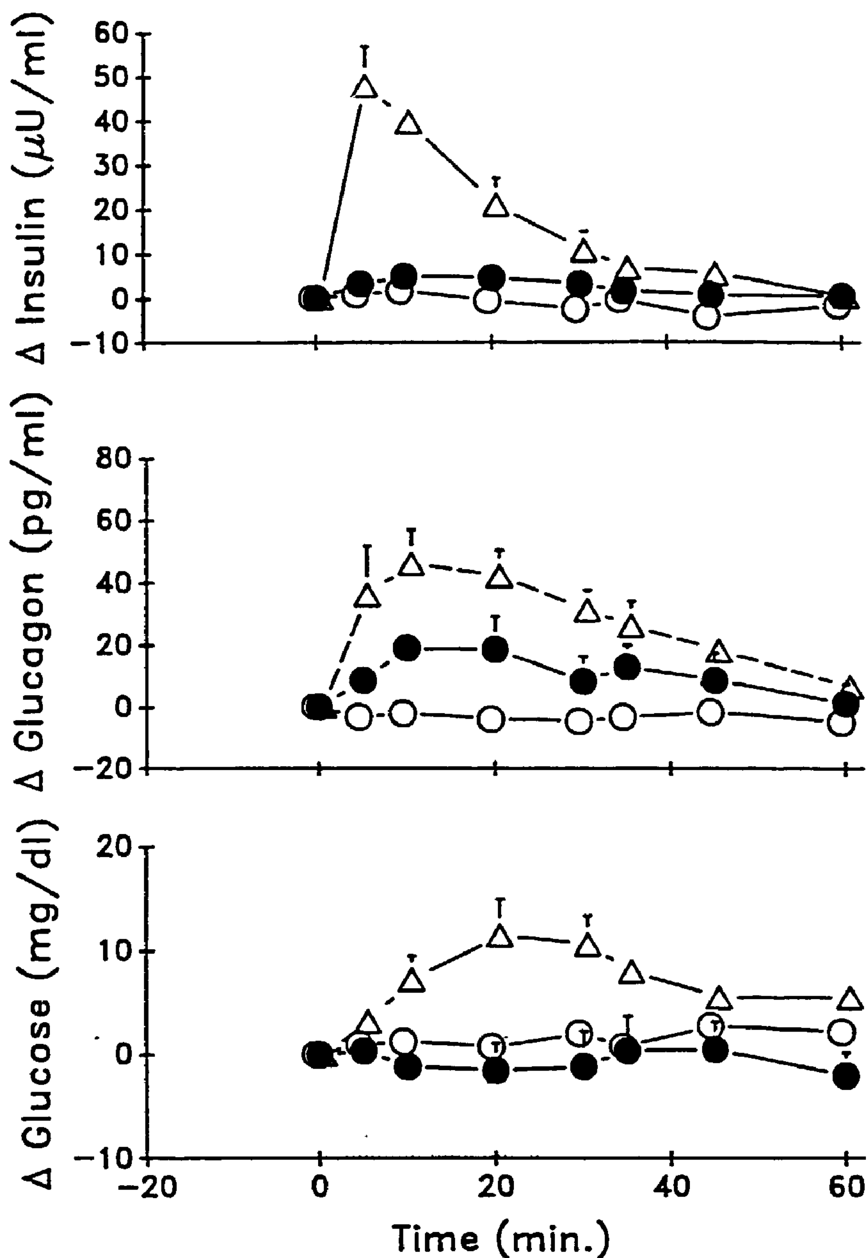

Figure 13. Mean ± SEM of the change over baseline of plasma insulin (top panel), plasma glucagon (middle panel), and plasma glucose (bottom panel) concentrations following a 30 minute infusion of (○) saline, (●) 100, and (△) 600 pmol/kg·hr of GRP into five non pregnant sheep that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

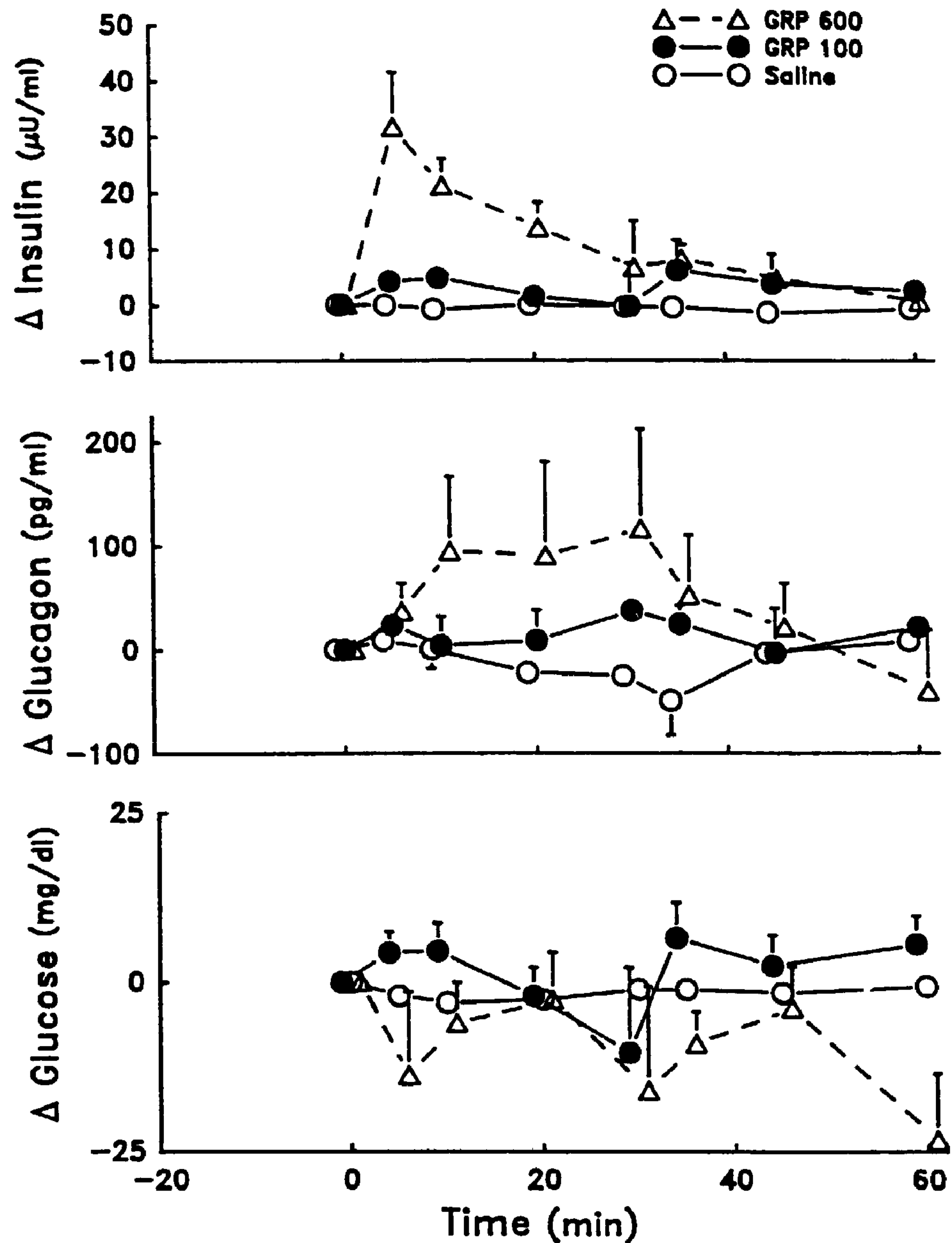

Figure 14. Mean ± SEM of the change over baseline of(top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (○) saline, (●) 100, and (Δ) 600 pmol/kg·hr of GRP into four pregnant ewes between d133-141 of gestation that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

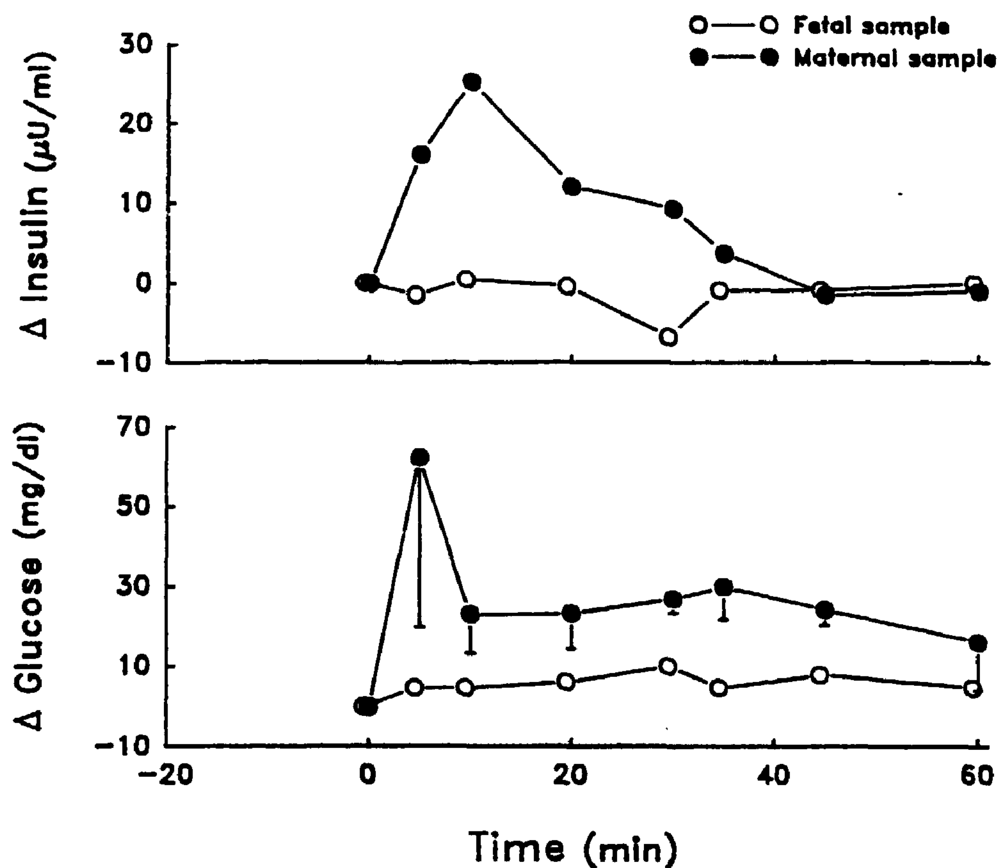

Figure 15. Mean ± SEM of the change over baseline of (top panel) plasma insulin and (bottom panel) plasma glucose concentrations measured in (●) two pregnant ewes and (○) their fetuses following a 30 minute infusion of 600 pmol/kg·hr of GRP administered to the pregnant ewe.

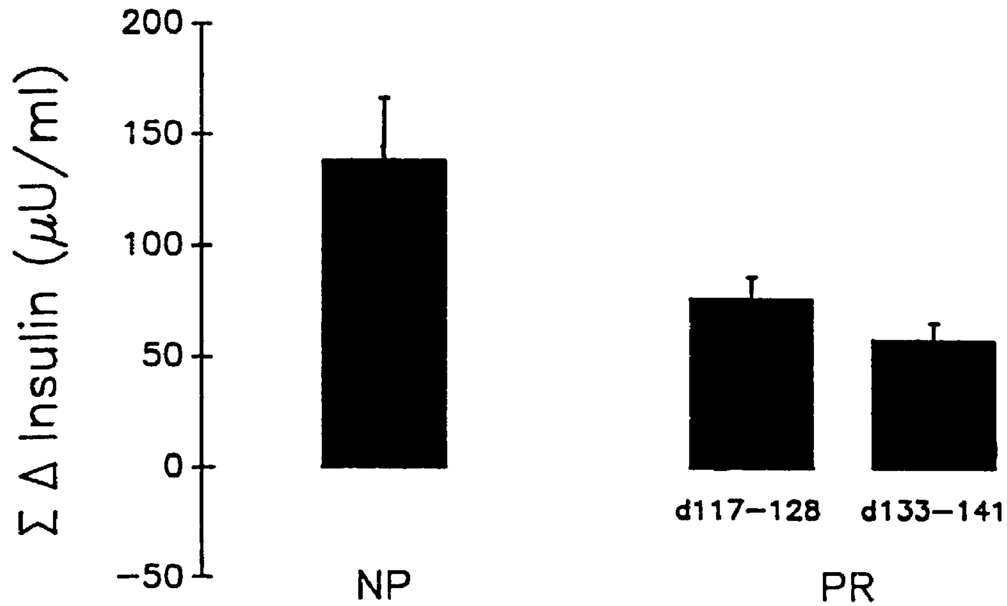

Figure 16. Bar graph of the mean ± SEM of the sum of the change over baseline in plasma insulin concentrations that occurred in response to a 30 minute intravenous infusion of 600 pmol/kg·hr of GRP into pregnant sheep (PR) at different times in gestation (d117-128; n=4, d133-141; n=4), and into adult non pregnant sheep (NP; n=5).

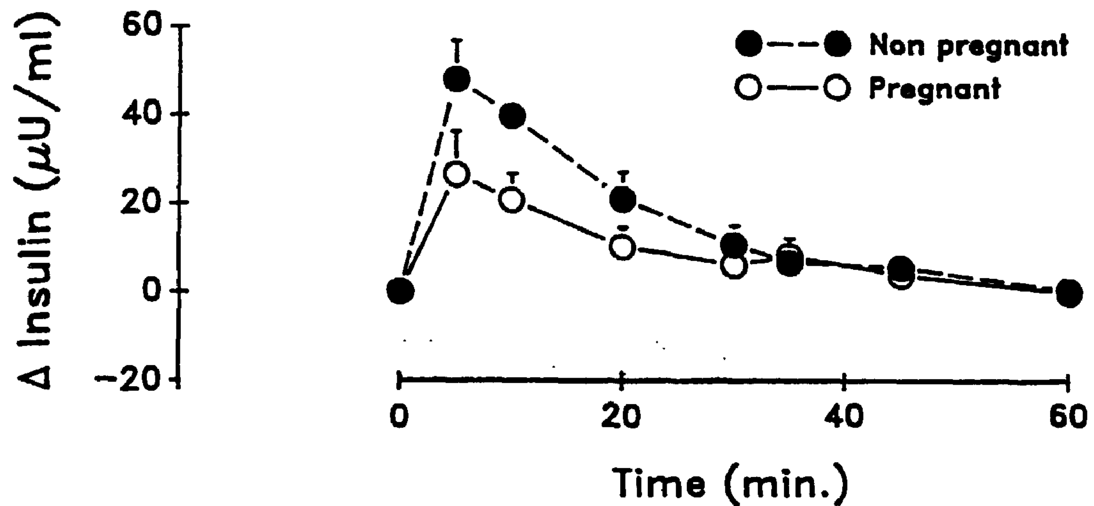

Figure 17. Mean ± SEM of the change over baseline of plasma insulin following a 30 minute infusion of 600 pmol/kg·hr of GRP into (○) pregnant; n=8 and (●) non pregnant; n=8 sheep that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

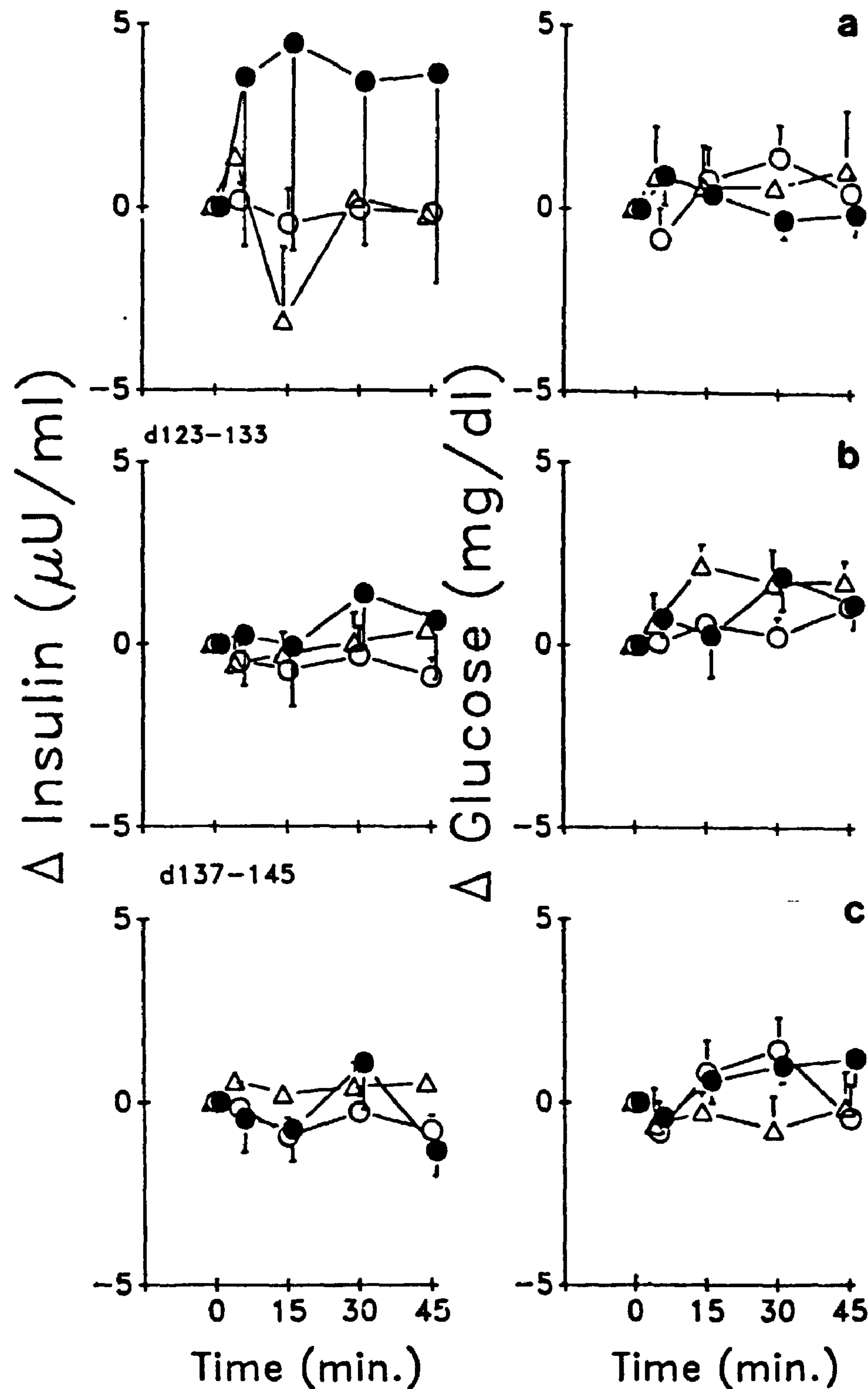

Figure 18. Mean ± SEM of the change over baseline of (right) plasma glucose and (left) plasma insulin following a 30 minute intravenous infusion (O) saline, (●) 100, and (Δ) 600 pmol/kg·hr of GRP in fetal lambs between (a) d110-117, n=4; (b) d123-133, n=11; and (c) d137-145, n=5; of gestation. (Where no SEM is shown, error is less than the size of the data point).

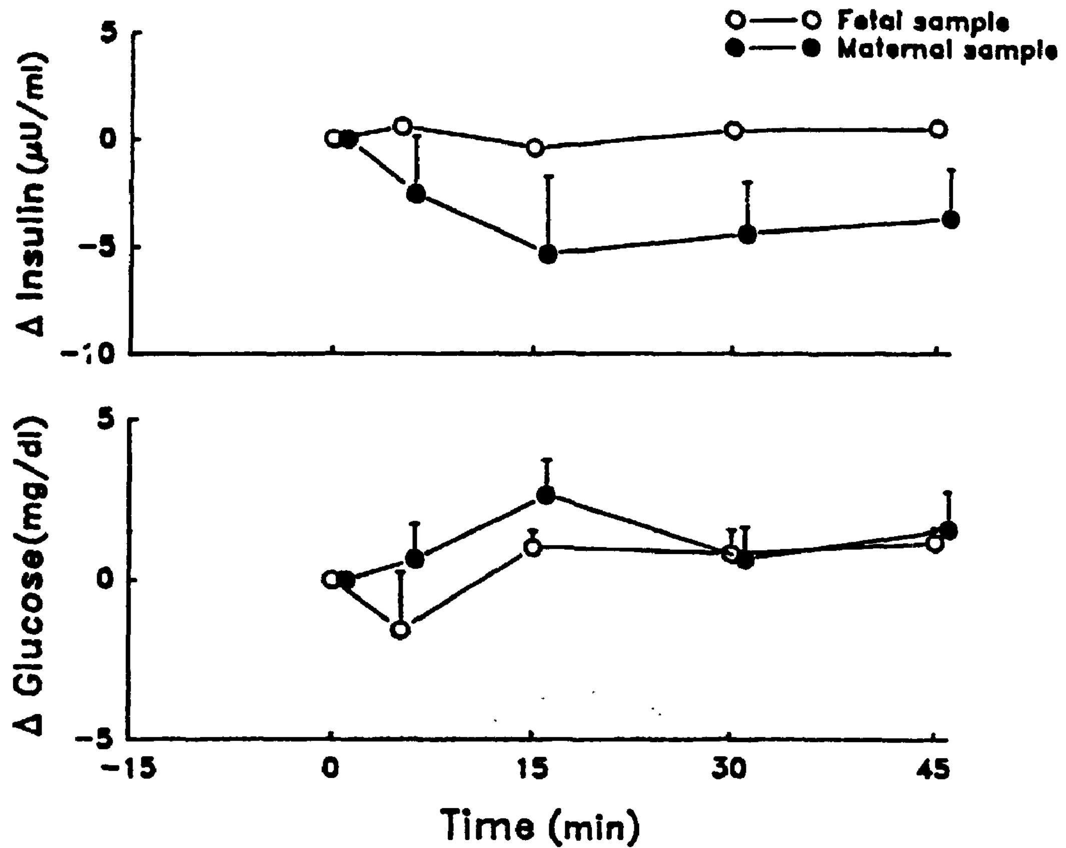

Figure 19. Mean ± SEM of the change over baseline of (top panel) plasma insulin and (bottom panel) plasma glucose concentrations measured in (●) three pregnant ewes and (O) their fetuses following a 30 minute infusion of 600 pmol/kg·hr of GRP administered to the fetuses.

7. INSULIN RESPONSE TO GRP IN FETAL, ADULT NON-PREGNANT, AND PREGNANT SHEEP: I. MODULATION BY GLUCOSE

7.1. INTRODUCTION

Previous studies performed in man (Wood et al., 1983) and rat (Greeley and Thompson, 1984; Martindale et al., 1982) demonstrate that GRP stimulates insulin release in a glucose dependent manner. Bloom et al (1984) have also shown that the ability of GRP to stimulate insulin release was influenced by circulating glucose and amino acid concentrations in conscious calves. The next series of experiments examined the hypothesis that the insulinotrophic effect of GRP in adult non-pregnant sheep would be attenuated when endogenous glucose concentrations are reduced by overnight fasting and that elevating circulating glucose concentrations via exogenous glucose administration would augment the insulin response to GRP. Further, it has been suggested previously that the impaired glucose tolerance which occurs during pregnancy may be due to the lack, or reduction in, the action of potentiators of glucose-stimulated insulin release (Hornnes et al., 1978). Therefore, we also examined whether GRP stimulates insulin release in a glucose dependent manner in pregnant sheep and whether the ability for GRP to alter glucose-stimulated insulin release is attenuated in pregnant compared to non-pregnant sheep.

Despite the fact that GRP administration into adult sheep stimulates insulin release previous studies presented in this thesis have demonstrated that administration of similar doses of GRP does not affect insulin release in fetal sheep between d110 to d145 of gestation (section 6.5.3). Circulating glucose concentrations in fetal sheep are significantly less than those in adult pregnant and non-pregnant sheep (section 6.5.1). It is therefore possible that the relative hypoglycemia present in fetal sheep is responsible for the lack of insulin response to GRP in fetal sheep. We therefore examined whether GRP is

capable of influencing insulin concentrations in fetal sheep in which circulating glucose concentrations are increased by concurrent exogenous glucose administration.

In summary, the purpose of the next series of experiments was to determine if the insulinotrophic effect of GRP in adult non-pregnant and pregnant sheep is glucose dependent and if the ability of GRP to potentiate glucose-stimulated insulin release is attenuated in pregnant compared to non-pregnant sheep. It was also determined whether the lack of insulin response to GRP in fetal sheep can be explained, at least in part, by low plasma glucose concentration present during fetal life. The following specific questions were addressed (1) Does overnight fasting attenuate the insulin response to GRP in adult non-pregnant and pregnant sheep? (2) Does exogenous glucose administration increase GRP-stimulated insulin release in adult non-pregnant sheep and pregnant sheep? (3) Is the ability of GRP to augment glucose-stimulated insulin release attenuated in pregnant compared to non-pregnant sheep? (4) Does increasing circulating glucose concentrations in fetal sheep by exogenous glucose administration result in a fetal insulin response to GRP?

7.2. MATERIAL AND METHODS

7.2.1. Animals

The following experiments were conducted in the same five non-pregnant ovariectomized sheep and eleven pregnant sheep used in the experiments described in the previous section (section 6.2.1). These sheep had previously had catheters surgically implanted into the femoral artery and vein as described (section 3.2). Unless otherwise indicated, animals were fed *ad libitum* as described previously (section 6.2.1).

7.2.2. Effect of Overnight Fasting

a) Experiments in Non-pregnant Ovariectomized Sheep

Five adult non-pregnant ovariectomized sheep were given GRP at doses of 0 (SALINE), 100 (GRP100), and 600 (GRP600) pmol/kg.hr as a 30 min. infusion via the femoral venous catheter. GRP was prepared and administered as described previously in section 6.22. Each treatment was given on separate days between 1200 hr and 1600 hr and the order of the treatments was randomized. In addition to experiments performed in non-pregnant sheep fed *ad libitum*, each treatment was repeated in the same animals following overnight fasting. On the day prior to an experiment in "fasted" non-pregnant sheep, the animals were fed at 0800 hr as previously described (section 6.2.1) but at 1500 hr when the animals would usually be fed again, these animals received only fresh water. Food was withheld overnight and the following day while the experiments which examined the effect of overnight fasting were conducted. In both fed and fasted non-pregnant sheep, blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +10, +20, +30, +35, +45, and +60 min. relative to the onset of the infusion. Plasma glucose, insulin, and glucagon concentrations were determined as described previously (section 3.5 & 3.6).

b) Experiments in Pregnant Sheep

Experiments similar to those described above in non-pregnant sheep (section 7.2.2a), were also conducted in a total of eleven pregnant sheep between d117 and 141 of gestation. GRP was administered at the same doses (SALINE, GRP100, and GRP600) as a 30 min. infusion via the maternal femoral venous catheter. Test substances were prepared and administered as described previously (section 6.22). Each test substance was administered to four d117-123 pregnant ewes fed *ad libitum* and in animals following overnight fasting. In addition, the experiments were performed in five "fed" and "fasted"

pregnant sheep at a later gestational age (between d129 to d141 of gestation). Experiments were repeated in certain pregnant ewes at more than one gestational age. The feeding regimes in the "fed" and "fasted" pregnant animals was similar to that described above for non-pregnant sheep (section 6.22). Maternal arterial blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +10, +20, +30, +35, +45, and +60 min. relative to the onset of the infusion. In experiments performed on three of the pregnant sheep, both fetal and maternal arterial blood samples were collected at all the times indicated above. Plasma glucose, insulin, and glucagon concentrations were determined as described previously (section 3.5 & 3.6).

7.2.3. Effect of Exogenous Glucose Administration

a) Experiments in Non-pregnant Ovariectomized Sheep

The following test substances were administered at a rate of 2ml/min for 30 min. into the femoral venous catheter of the same adult non-pregnant ovariectomized sheep; 10 mg/kg.min of glucose given alone (Glu), 10 mg/kg.min of glucose given together with 100 pmol/kg.hr of GRP (Glu & GRP100), and 10 mg/kg.min of glucose given together with 600 pmol/kg.hr of GRP (Glu & GRP600). In a preliminary study, exogenous administration of 10 mg/kg.min of glucose was determined to submaximally stimulate insulin release from fetal and non-pregnant sheep (data not shown). Experiments were performed on separate days in a randomized order between 1200 hr and 1600 hr.

Test substances containing glucose were prepared in the following manner. A sufficient amount of D-glucose to obtain the desired doses indicated above, was dissolved in saline to give a final volume of 65 ml. The solution was then drawn up into a 60 ml syringe and passed through a 0.8 μm and then a 0.2 μm filter (Millipore Corp, Bedford, MA) into a sterile container. Using sterile techniques the infusate was then transferred into another sterile 60 ml syringe which was used to administer the test substance. In

experiments where GRP and glucose were administered together, a sufficient amount of GRP necessary to obtain the desired dose, was dissolved in 1-5 ml of sterile saline and drawn up into a 5 ml syringe. Using a sterile 18 gauge needle, the GRP solution was then added to the syringe containing the previously sterilized glucose solution. The syringe was then inverted several times to insure proper mixing. Glucose solutions were prepared within an hour before experiments commenced and kept on ice. If GRP was administered with the glucose, it was added just prior to (5-10 min.) administration.

Blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +10, +20, +30, +35, +45, and +60 min. relative to the onset of the infusion. Plasma glucose, insulin, and glucagon concentrations were determined as described previously (section 3.5 & 3.6).

b) Experiments In Pregnant Sheep

Experiments similar to those described above in non-pregnant sheep (section 7.22b), were also carried out in eight d117-141 pregnant sheep which were fed *ad libitum*. A 30 min. infusion of either Glu, Glu & GRP100, or Glu & GRP600 was prepared and administered via the femoral venous catheter, as described previously (section 7.23a). Maternal arterial blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +10, +20, +30, +35, +45, and +60 min. relative to the onset of the infusion. In three of the experiments performed in pregnant sheep between d133 and 141 of gestation, fetal arterial blood samples were also collected at the times indicated above. Plasma glucose, insulin, and glucagon concentrations were determined as described previously (section 3.5 & 3.6).

7.2.4. Experiments In Fetal Sheep

Similar experiments were also performed on the healthy (pO_2>20mmHg, pCO_2=40-50 mmHg, pH=7.30-7.35) fetuses of the above mentioned pregnant ewes. A 30 min.

infusion of either GLU, GRP100 & GLU, or GRP600 & GLU was administered via the fetal femoral venous catheter at a rate of 0.2 ml/min. for 30 min., as described previously (section 7.2.3a). Test substances were prepared as described previously (section 7.22b) in 20 ml of sterile saline at similar doses based on estimated fetal body weight (2.25 kg at d117- 130; 3.5 kg at d130-141 of gestation; Barcroft, 1945). Fetal and maternal arterial blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +15, +30, and +45 min. relative to the onset of the infusion. Fetal and maternal plasma glucose and insulin concentrations and maternal plasma glucagon concentrations were determined as described previously (section 3.5 & 3.6). In addition, in order to assess fetal health throughout the infusion of the test substance, a further 0.25 ml of blood was collected at each time point and fetal blood gases (pO_2, and pCO_2) and pH were determined.

7.2.5. Data Analysis

The results obtained from GRP administration to non-pregnant and pregnant sheep that were fed *ad libitum* were described in detail in the previous chapter and are included in this chapter for the purpose of comparison.

Results are presented as the mean ± SEM of the change over the mean of the two pre-injection values for the number of animals indicated (n). The effect of time and treatment on glucose, glucagon, and insulin concentrations in plasma was assessed using a nested analysis of variance where numbers in treatment groups were equal, and using a two way unbalanced analysis of variance where there were unequal numbers between treatment groups. Multiple comparisons were then performed using Duncan Multiple Range test.

The sum changes in plasma insulin concentrations in fetal and adult sheep during GRP administration were calculated as the mean ± SEM of the summed increases over the average of the two pre-injection values for each individual animal. One way analysis of variance was used to test for significant differences in the sum change in plasma insulin, glucagon, and glucose concentrations between fetal, pregnant and non-pregnant sheep. Basal plasma glucose, insulin, and glucagon concentrations were calculated and analyzed statistically as described previously (section 6.2.5). Differences were considered statistically significant at $p<0.05$.

7.3. RESULTS

7.3.1. Basal Concentrations

When pregnant ewes at d117 - 123 of gestation were fasted overnight, basal glucose and insulin concentrations were significantly reduced compared to levels measured in animals at a similar gestational age that were fed *ad libitum* (refer to Table 2). Correspondingly, plasma glucagon concentrations were significantly greater ($p<0.05$) and plasma insulin concentrations were significantly lower ($p<0.05$) in fasted compared to fed d117-123 pregnant sheep. In pregnant sheep examined later on in gestation (d129-141), plasma glucose concentrations were significantly reduced by overnight fasting but this was not associated with any significant change in either plasma insulin or glucagon concentrations (Table 2).

Following overnight fasting, non-pregnant sheep had significantly lower basal plasma glucose concentrations ($p<0.05$) but this was not associated with any significant difference in basal plasma insulin concentrations between fed and fasted non-pregnant sheep (Table 2).

7.3.2. Effect of Overnight Fasting

a) Experiments in Non-pregnant Ovariectomized Sheep

Infusion of GRP600 into non-pregnant sheep which were fasted overnight, significantly increased plasma insulin concentrations over saline treated controls at +5 and +10 min. after the onset of the GRP infusion ($p<0.05$; Fig. 20). Peak insulin elevations of 34 ± 9 µU/ml occurred at +5 min. and subsequently declined, despite continued GRP administration. Administration of the lower dose of GRP (GRP100) did not significantly change plasma insulin concentrations in fasted non-pregnant sheep (Fig. 20). In addition no statistically significant change in plasma glucose concentrations occurred in response to the administration of either dose of GRP (Fig. 20). There was no significant difference between the plasma insulin elevations at individual time points during the infusion of GRP600 into non-pregnant sheep fed *ad libitum* compared to the same animals tested following overnight fasting (Fig. 21).

b) Experiments in Pregnant Sheep

Administration of the higher dose of GRP (GRP600) into d117-123 fasted pregnant ewes resulted in small but significant, transient, elevation in plasma insulin concentrations (6 ± 2 µU/ml at +5 min.; $p<0.05$), more gradual but sustained increases in plasma glucagon concentrations (27 ± 16 µU/ml at +30 min.; $p<0.05$), but no significant change in plasma glucose concentrations (Fig. 22). The infusion of the lower dose of GRP (GRP100) into pregnant ewes following overnight fasting had no significant effect on plasma insulin, glucagon, and glucose concentration (Fig. 22). Changes in plasma insulin, glucagon, and glucose concentrations in response to either dose of GRP were not significantly different in fasted pregnant sheep tested between d117-123 of gestation compared to those tested later in gestation (d129-141; Fig. 23).

Plasma insulin elevations in response to GRP administration were markedly less in pregnant sheep fasted overnight (4 ± 2 µU/ml at +5 min.) compared to the same animals fed *ad libitum* (23 ± 8 µU/ml at 5min; Fig. 24; $p<0.05$). Peak plasma glucagon elevations occurred at +10 min. following administration of GRP600 and not significantly different between fed (61 ± 36 pg/ml; Fig. 14) and fasted (52 ± 16 pg/ml; Fig. 22) pregnant sheep. GRP administration did not produce a significant change in plasma glucose concentrations in either fed (Fig. 14) or fasted (Fig. 22) pregnant sheep.

c) Comparison of the Effect of Overnight Fasting Between Pregnant and Non-pregnant Sheep

The insulin response, expressed as the sum of the change over baseline, to the administration of the same dose of GRP (GRP600) was significantly greater in fed (139 ± 28 µU/ml) compared to fasted (61 ± 21 µU/ml) non-pregnant sheep (refer to Fig. 25; $p<0.05$). The sum change of insulin during GRP100 administration was also attenuated following overnight fasting of pregnant and non-pregnant sheep but these differences were not statistically different (Fig. 25).

7.3.3. Effect of Exogenous Glucose Administration

a) Experiments in Non-pregnant Ovariectomized sheep

Exogenous glucose administration (Glu) into non-pregnant sheep ovariectomized sheep resulted in a gradual increase in plasma glucose concentrations with peak concentrations of 86 ± 4 mg/dl occurring at +30 min. ($p<0.05$; Fig. 26). Plasma glucose concentrations declined when the 30 min. glucose infusion was completed but remained significantly above baseline at +60 min. (Fig. 26; $p<0.05$). Similar changes in plasma glucose concentrations were produced following administration of glucose alone, or glucose together with either GRP100 (Fig. 26) or GRP600 (Fig. 27).

During the infusion of glucose alone, plasma insulin concentrations were significantly elevated above baseline by +5 min. (23 ± 7 µU/ml), increased to peak insulin concentrations of 50 ± 9 µU/ml by 30 min. and then declined to baseline (Fig. 26). Glucose administration together with GRP100 resulted in plasma insulin elevations at +10 min. to +45 min. which were significantly greater than those which occurred in response to glucose administration alone ($p<0.05$). Peak insulin elevations of 126 ± 41 µU/ml occurred at 10 min., remained elevated for the duration of the 30 min. and then subsequently declined to baseline following the offset of the Glu & GRP100 infusion (Fig. 26). Similar trends were also observed when glucose was infused together with the higher dose of GRP (GRP600; Fig. 27). Peak plasma insulin elevations were significantly greater following administration of Glu & GRP600 (177 ± 43 µU/ml; $p<0.05$) compared to those which occurred when glucose was infused alone (Glu; 50 ± 19 µU/ml; Fig. 27).

Plasma glucagon concentrations were significantly elevated over baseline at +10 min. after the onset of the Glu & GRP100 infusion (Fig. 26; $p<0.01$) and between +5 min. and +20 min. after Glu & GRP600 was administered (Fig. 27; $p<0.05$). Peak glucagon elevations following administration of Glu & GRP100 (41 ± 12 pg/ml) were significantly less than those following GRP100 (82 ± 20 pg/ml; $p<0.05$) but were similar to those following Glu administration (11 ± 19 pg/ml; Fig. 26). When glucose was administered together with the higher dose of GRP600, peak plasma glucagon concentrations (58 ± 19 pg/ml) were not significantly different than those measured following administration of GRP600 (61 ± 18 pg/ml) but were significantly greater than the insignificant changes in plasma glucagon concentrations that occurred during the infusion of glucose alone (11 ± 19 pg/ml; Fig. 27; $p<0.05$). Therefore, concurrent exogenous glucose administration reversed the ability for the lower dose of GRP (GRP100) to stimulate glucagon release but did not alter the glucagon response to GRP600.

b) Experiments in Pregnant Sheep

Peak glucose concentrations were similar in pregnant sheep administered Glu (128 ± 13 mg/dl) or Glu & GRP100 (128 ± 15 mg/dl). In both cases plasma glucose levels were significantly increased over baseline throughout the infusion of glucose with and without GRP and remained elevated in the last sample taken 30 min. after the offset of the infusion ($p<0.05$; Fig. 28). Peak plasma glucose concentrations achieved during the infusion Glu & GRP600 (81 ± 17 mg/dl) were significantly less than those which occurred following administration of Glu (128 ± 13 mg/dl; $p<0.05$) or Glu & GRP100 (128.2 ± 15.2 mg/dl; Fig. 28 & 29; $p<0.05$). In addition, plasma glucose levels returned to baseline more rapidly following the administration of Glu & GRP600 (Fig. 29) compared to those following administration of Glu and Glu & GRP100 (Fig. 28; $p<0.05$).

In pregnant sheep, the infusion of either Glu or Glu & GRP100 resulted in gradual increases in plasma insulin concentrations with statistically significant peak insulin elevations of 31 ± 10 µU/ml and 61 ± 25 µU/ml, respectively, occurring at the end of the 30 min. infusion (Fig. 28; $p<0.05$). Plasma insulin concentrations remained significantly elevated above baseline ($p<0.05$) in the last sample taken at + 60 min. in response to the infusion of Glu and Glu & GRP100 (Fig. 28) but rapidly declined to baseline following the administration of Glu & GRP600 (Fig. 29). Peak elevations in plasma insulin concentrations were not significantly different between pregnant sheep given Glu & GRP100 (61 ± 25 µU/ml; Fig. 28), Glu & GRP600 (32 ± 23 µU/ml; Fig. 29) and Glu (31 ± 10 µU/ml; Fig. 28 & 29).

Maternal plasma glucagon concentrations were not significantly altered during exogenous glucose adminstration. Plasma glucagon concentrations were significantly elevated above baseline at +30 to +35 min. relative to the onset of the GRP100 infusion (Fig. 28) and during and after the administration of GRP600 (Fig. 29; $p<0.05$). However

when either dose of GRP was administered together with glucose there was no significant change in plasma glucagon concentrations (Fig. 28 & 29).

c) Effect of Maternal Exogenous GRP and/or Glucose Infusion on the Fetus

Changes in plasma concentrations of insulin, glucagon, and glucose during the infusion of Glu, Glu & GRP100, and Glu & GRP600 into the maternal venous catheter were determined in two or three fetuses (Fig. 30). As a result, no significant change was demonstrable. It is apparent however, that administration of GRP and/or glucose to the pregnant ewe resulted in changes in plasma glucose and insulin concentrations in the fetal compartment which mimicked the maternal responses described above (refer to Fig. 28 & 29).

d) Comparison of the Insulin Responses to GRP and/or Glucose Between Non-pregnant and Pregnant Sheep

The insulin response, expressed as the sum change over baseline of plasma insulin concentrations, to the infusion of GRP600 was significantly less in pregnant compared to non-pregnant ovariectomized sheep (Fig. 31; $p<0.05$). Furthermore, the insulin response to glucose given together with either dose of GRP was also significantly less in pregnant compared to non-pregnant sheep (Fig. 31; $p<0.05$). In non-pregnant sheep, concurrent administration of glucose and GRP100 (Glu & GRP100) produced a sum change in plasma insulin concentrations of 792 ± 292 μU/ml which was significantly greater than responses to either GRP100 alone 7 ± 8 μU/ml; $p<0.05$) or glucose alone (231 ± 74 ± μU/ml; p<0.,05). Furthermore, the insulin response to GRP100 & Glu also was significantly greater than sum of the individual responses (Glu + GRP100 = 212 ± 71 μU/ml) which demonstrates that GRP potentiates glucose-stimulated insulin release in adult non-pregnant sheep. However, in pregnant sheep, the insulin response to glucose given separately (149 ± 40 μU/ml) or together with either GRP100 (261 ± 89 μU/ml) or

GRP600 (148 ± 119 μU/ml) were not significantly different. Therefore the potentiative effect of GRP on glucose-stimulated insulin release is not present in pregnant sheep.

Glucose administration tended to decrease plasma glucagon concentrations and attenuate GRP-stimulated glucagon release in both pregnant and non-pregnant sheep (Fig. 31). However, due to large variations in individual values there was no significant difference between the glucagon responses in non-pregnant compared to pregnant sheep.

Plasma glucose concentrations were increased significantly when GRP600 was administered to non-pregnant but not pregnant sheep (Fig. 31; $p<0.05$). Plasma glucose elevations were significantly greater following exogenous glucose administration to pregnant (689 ± 70 mg/dl) compared to non-pregnant sheep (397 ± 34 mg/dl; Fig. 31; $p<0.05$). In both pregnant and non-pregnant sheep, plasma glucose elevations were not significantly different following administration of glucose alone compared to glucose administration together with either dose of GRP. There was no significant increase in plasma glucose concentrations following the administration of GRP at a dose of 100 pm/kg.hr to non-pregnant and pregnant sheep (Fig. 31).

7.3.4. Experiments in Fetal Sheep

Exogenous glucose administration to fetal sheep resulted in significant elevations in circulating plasma glucose concentrations of 11 ± 2 mg/dl at +15 min. (Fig. 32; $p<0.05$). Fetal plasma glucose concentrations remained elevated during the 30 min. infusion and then subsequently declined. There was no significant difference between peak plasma glucose elevations which occurred in fetuses following the administration of Glu (11 ± 2 mg/dl), Glu & GRP100 (10 ± 1 mg/dl; Fig. 32), or Glu & GRP600 (21 ± 9 mg/dl; Fig. 33). In addition, fetal blood pO_2, pCO_2, pH was not altered during the infusion of glucose and/or GRP into the fetus.

Elevations in fetal plasma insulin concentrations were not significantly different following Glu (11 ± 2 μU/ml) Glu & GRP100 (10 ± 2 μU/ml; Fig. 32) or Glu & GRP600 (11 ± 5 μU/ml; Fig. 33) administration. The administration of either dose of GRP alone had no effect on plasma insulin or glucose concentrations measured in fetuses tested between d120-141 of gestation. Maternal plasma insulin and glucose concentrations were also not affected by the administration of either, Glu, Glu & GRP100, or Glu & GRP600 to the fetus (data not shown). Therefore increasing circulating glucose concentrations by concurrent exogenous glucose administration does not appear to alter the unresponsiveness of the fetal endocrine pancreas.

7.4. DISCUSSION

These studies demonstrated that the ability of GRP to stimulate insulin release is modulated by circulating glucose concentrations in adult non-pregnant and pregnant sheep. Overnight fasting of non-pregnant and pregnant sheep, which resulted in significant reduction in circulating glucose concentrations, attenuated the insulin response to GRP administration. The results presented here also demonstrate that GRP potentiates glucose-stimulated insulin release in non-pregnant sheep but not pregnant sheep. These results therefore support the hypothesis that reduced action of potentiators of glucose-stimulated insulin release, may, in part, explain impaired glucose tolerance which is associated with pregnancy. Furthermore, these studies have demonstrated that elevating circulating concentrations of glucose did not alter the lack of insulin response to the doses of GRP administered to fetal sheep.

Despite the fact that carbohydrate metabolism differs between ruminants and monogastric species (section 1.2) the present study demonstrated that pregnancy associated changes in circulating glucose and insulin concentrations, which have been

well documented in pregnant women (Baird, 1976), also occur in pregnant sheep. For example: (1) insulin concentrations in fed and fasted pregnant sheep increased with gestational age and were greater in d129 - 141 pregnant sheep compared to non-pregnant sheep and (2) overnight fasting produced a greater reduction in plasma glucose concentrations in pregnant compared to non-pregnant sheep, demonstrating a state of "accelerated starvation" during pregnancy. Several studies performed in pregnant women have demonstrated an impaired tolerance to oral and IV administration of glucose when compared to non-pregnant and post-partum women (Buch et al., 1986; Bender and Chickering, 1985; Baird, 1986). The present studies performed in pregnant sheep did not examine the effect of oral glucose administration but did demonstrate that IV administration of the same dose of glucose produced similar insulin responses but significantly greater elevations in glucose levels than responses in non-pregnant sheep. This suggests that pregnant sheep, like pregnant women, have impaired tolerance to IV glucose administration. Whether or not the insulin response to oral glucose administration is impaired in pregnant sheep remains to be determined.

Overnight fasting of non-pregnant sheep, which significantly reduced basal glucose concentration, attenuated significantly the insulin response (expressed as the sum change over baseline) to GRP administration when compared to the insulin response in the same sheep fed *ad libitum*. Similarly, reduced circulating glucose concentrations by overnight fasting abolished the ability for GRP to stimulate insulin release in fasting pregnant sheep. Conversely a greater insulin response to GRP was produced in non-pregnant sheep when circulating glucose concentrations were elevated by concurrent exogenous glucose administration. Collectively these results suggest that the insulin response to GRP is glucose dependent in pregnant and non-pregnant sheep. This is consistent with previous reports in monogastric species (Wood et al., 1983; Martindale et al., 1982; Greeley and Thompson, 1984) and other ruminants (Bloom et al., 1984).

In non-pregnant sheep, the insulin response, expressed as the sum change in plasma insulin concentrations over baseline, was significantly greater when GRP and glucose were administered together than the sum of the insulin responses to glucose given alone and GRP given alone. These results demonstrate that GRP is a potentiator of glucose-stimulated insulin release in non-pregnant sheep. This suggests that GRP and glucose act through different mechanisms to stimulate insulin release from the β-cell. The mechanisms by which glucose stimulates insulin release is of considerable debate. It is known that in order to elicit insulin secretion, glucose must enter the β-cell via facilitated diffusion (Hellman et al., 1971) and be oxidized (Grill and Cerasi, 1976). A glucose mediated event or combination of events associated with the oxidation of glucose within the β-cell stimulates three intracellular messenger systems: (1) activation of adenylate cyclase (Schuit and Pipleers, 1985), (2) calcium entry into the β- cell (Ashcroft, 1981; Malaisse and Mathias, 1985), and (3) increased phosphatidyl inositol turnover (Hedeskov, 1980). The mechanism of GRP action on peripheral targets is believed to involve: 1) mobilization of cellular calcium (Moody et al., 1987); 2) activation of protein kinase C (Muir and Murray, 1987; Brown et al., 1987); and 3) increased phosphatidyl inositol turnover (Brown et al., 1987; Lloyd et al., 1989). The anatomical proximity of GRP immunoreactive nerves to pancreatic islets in pigs (Ghatei et al., 1984) and dogs (Buchan and McDonald, unpublished results) suggests that GRP may act directly on islet function. This is supported by *in vitro* studies which have demonstrated that GRP stimulates insulin release from isolated islets (Greeley et al., 1986) and neoplastic β-cell line (Swope and Schonbrunn, 1984). Studies in conscious calves have detected GRP in the systemic circulation (Bloom et al., 1984). Therefore in ruminants, GRP may influence pancreatic function by acting in an endocrine fashion. GRP stimulates the release of several different GI hormones many of which are capable of affecting pancreatic islet function. As a result,

it is possible that the insulin response to IV administration of GRP in sheep observed in this study is mediated through the effects of GRP on the release of other insulinotrophic peptides.

The ability of GRP to potentiate glucose-stimulated insulin release was less in pregnant compared to non-pregnant sheep. The effect of GRP administration has not been examined during pregnancy in any species and whether or not the rate of metabolism of GRP is different during pregnancy is unknown. The placenta is capable of both producing and metabolizing other GI peptides such as gastrin and VIP (Shulkes et al., 1987). The role the placenta plays in GRP production and metabolism is unknown. During pregnancy there is a 10-30% increase in circulating blood volume compared to the non-pregnant state. Therefore it is possible that enhanced GRP metabolism and/or the dilution of GRP in a greater blood volume in pregnant sheep accounts, at least in part, for the attenuated insulin response to GRP and reduced ability for GRP to augment glucose-stimulated insulin release that was observed in pregnant compared to non-pregnant sheep. These issues will be addressed in a later section of this thesis (Chapter 9).

The action of other potentiators of insulin release such as GIP, motilin, somatostatin, and VIP are diminished during pregnancy (Jenssen et al., 1988) and the release of these peptides in response to feeding is attenuated in pregnant compared to post-partum women (Lorrain et al., 1977; Hornnes et al., 1978). The results in this study are consistent with the suggestion that reduced action of potentiators of insulin release may, at least in part, be responsible for impaired glucose tolerance during pregnancy.

Factors which have been implicated as mediators of the changes in metabolism during pregnancy include glucocorticoids, PRL, PL, and P_4 and E_2. These hormones have all been demonstrated to produce a similar kind of insulin resistance which occurs during pregnancy and circulating concentrations of these hormones become elevated during

pregnancy and rise progressively towards term. Evidence supporting the role of glucocorticoids (Hornnes and Kuhl, 1986), PRL (Landgraf et al., 1977) and PL (Beck and Daughaday, 1967, Grumbach et al., 1968; Beck, 1970) in mediating pregnancy-induced alterations in metabolism is unclear and often times conflicting. A role of P_4 and/or E_2 in this regard seems more likely. Both structural (Aerts et al., 1980) and functional changes (Baird, 1976; Bender and Chickering, 1985) in the endocrine pancreas associated with pregnancy have been reproduced by exogenous administrations of physiological amounts of P_4 and/or E_2 to non-pregnant adults in many different species. The mechanism by which these steroids exert these effects is unknown. It is also unknown whether elevated concentrations of P_4 and/or E_2 play a role in mediating altered insulinotropic actions of GI peptides such as GRP which has been reported to occur during pregnancy. These questions have been addressed in experiments described in the following chapter (Chapter 8).

At the amounts given, GRP does not appear to influence either insulin secretion or glucose-stimulated insulin release in fetal sheep. Since studies presented here demonstrated that a reduction in endogenous glucose concentrations due to overnight fasting abolished the insulin response to GRP in pregnant sheep, it is possible that lower circulating glucose concentrations present in the fetus are responsible for the lack of insulin response to GRP observed in fetal sheep. However, concurrent exogenous glucose administration to the fetus did not alter the unresponsiveness of the fetal endocrine pancreas to GRP. Although exogenous glucose administration did significantly increase fetal plasma glucose concentrations, the concentrations produced were less than basal glucose concentrations in adult sheep fed *ad libitum.* Glucose consumption by the fetus and placenta accounts for a substantial portion of oxygen consumption in the fetus (Hay and Sparks, 1985). As a result, exogenous glucose administration can produce

significant reductions in fetal PO_2 levels and if administered in sufficiently high quantities, it can cause fetal death. In addition, hypoxia and other stresses abolish glucose-stimulated insulin release in sheep (Fowden, 1985; Jones et al., 1988). In the present experiments, glucose was administered in quantities sufficient to cause a significant increase in fetal plasma glucose concentrations without producing a change in either fetal $P0_2$ or $PC0_2$ levels.

The lack of change in fetal plasma insulin concentrations in response to exogenous GRP administration suggests that in the fetal sheep endocrine pancreas is unresponsive to GRP. Tissue and plasma concentrations of GRP need to be examined in fetal sheep. It is also not known whether the metabolism and production of GRP is similar in fetal and adult sheep. It is possible therefore that a similar dose per kg body weight of GRP administered to fetal and adult sheep resulted in circulating concentrations in the fetus which were insufficient to stimulate insulin secretion from the fetal endocrine pancreas. This question has been addressed in Chapter 9. It is also possible the mechanism by which GRP stimulates insulin release in the adult is functionally immature in fetal sheep and does not develop until after birth.

In conclusion, these studies have demonstrated that GRP-stimulated insulin release is glucose dependent in adult non-pregnant and pregnant sheep but not fetal sheep and that the ability of GRP to potentiate glucose-stimulated insulin release is less in pregnant sheep compared to non-pregnant sheep. The results support the hypothesis that impaired glucose tolerance which occurs during pregnancy may be mediated, at least in part, by the reduced action of potentiators of glucose-stimulated insulin release, su h as GRP. Factors which may mediate the reduced insulinotropic action of GRP in pregnant sheep are the subject of the next chapter.

	Glucose (mg/dl)	Insulin (μU/ml)	Glucagon (pg/ml)
PREGNANT SHEEP			
FASTED			
d117–123	53 ± 1	7 ± 3	134 ± 17
d129–141	50 ± 1	19 ± 2	104 ± 25
FED			
d117–128	72 ± 5	14 ± 2	62 ± 13
d133–141	72 ± 3	22 ± 2	95 ± 17
NON PREGNANT SHEEP			
FASTED	53 ± 2	10 ± 1	----
FED	60 ± 1	12 ± 1	112 ± 8

Table 2. Mean ± SEM of plasma insulin, glucose, and glucagon concentrations measured in preinjection samples collected from non pregnant sheep and pregnant sheep at different gestational ages that were fed *ad libitum* and in the same animals following overnight fasting.

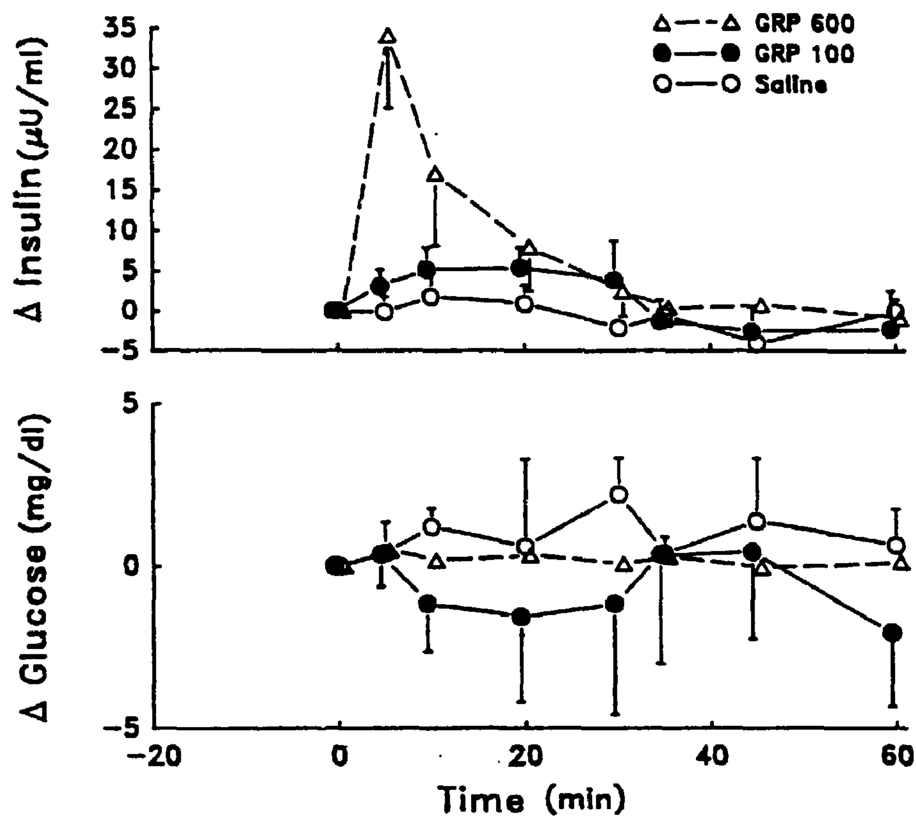

Figure 20. Mean ± SEM of the change over baseline of (top panel) plasma insulin and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (○) saline, (●) 100, and (△) 600 pmol/kg·hr of GRP into five non pregnant ewes following overnight fasting.

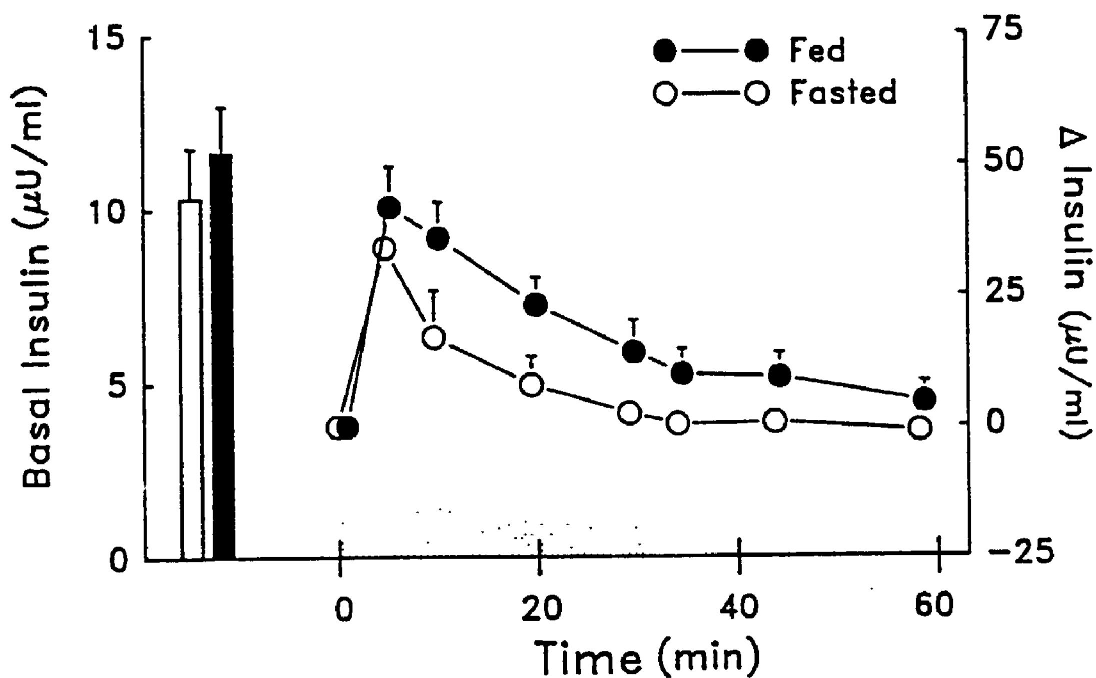

Figure 21. Mean ± SEM of basal insulin concentrations (left histogram) and the change over baseline of plasma insulin concentrations during the infusion of 600 pmol/kg·hr of GRP (right axis) into non pregnant sheep (●) fed *ad libitum*; n=5, and (O) following overnight fasting; n=8. (Where no SEM is shown, error is less than the size of the data point).

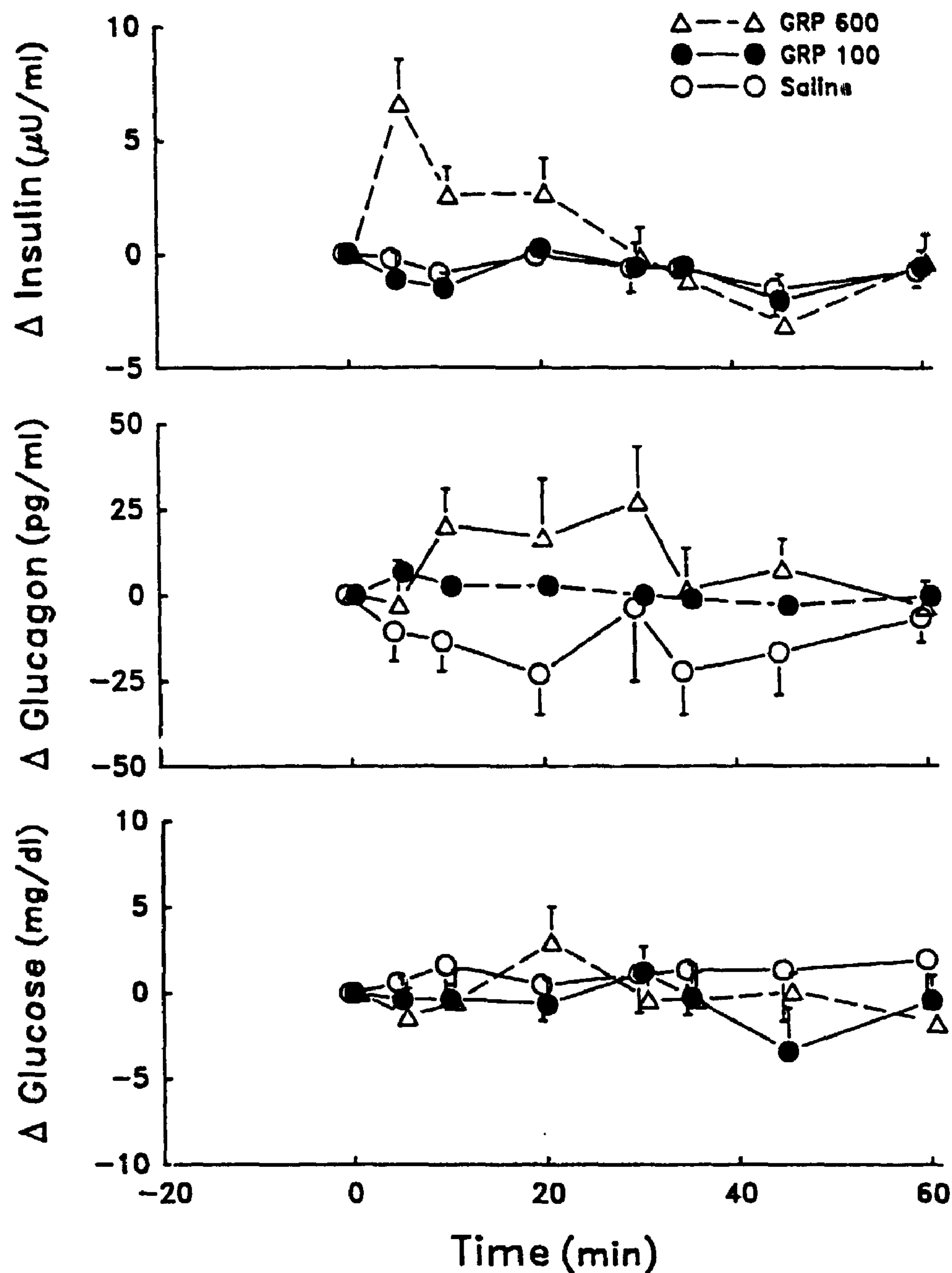

Figure 22. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (○) saline, (●) 100, and (△) 600 pmol/kg·hr of GRP into five pregnant ewes between d117-123 of gestation following overnight fasting. (Where no SEM is shown, error is less than the size of the data point).

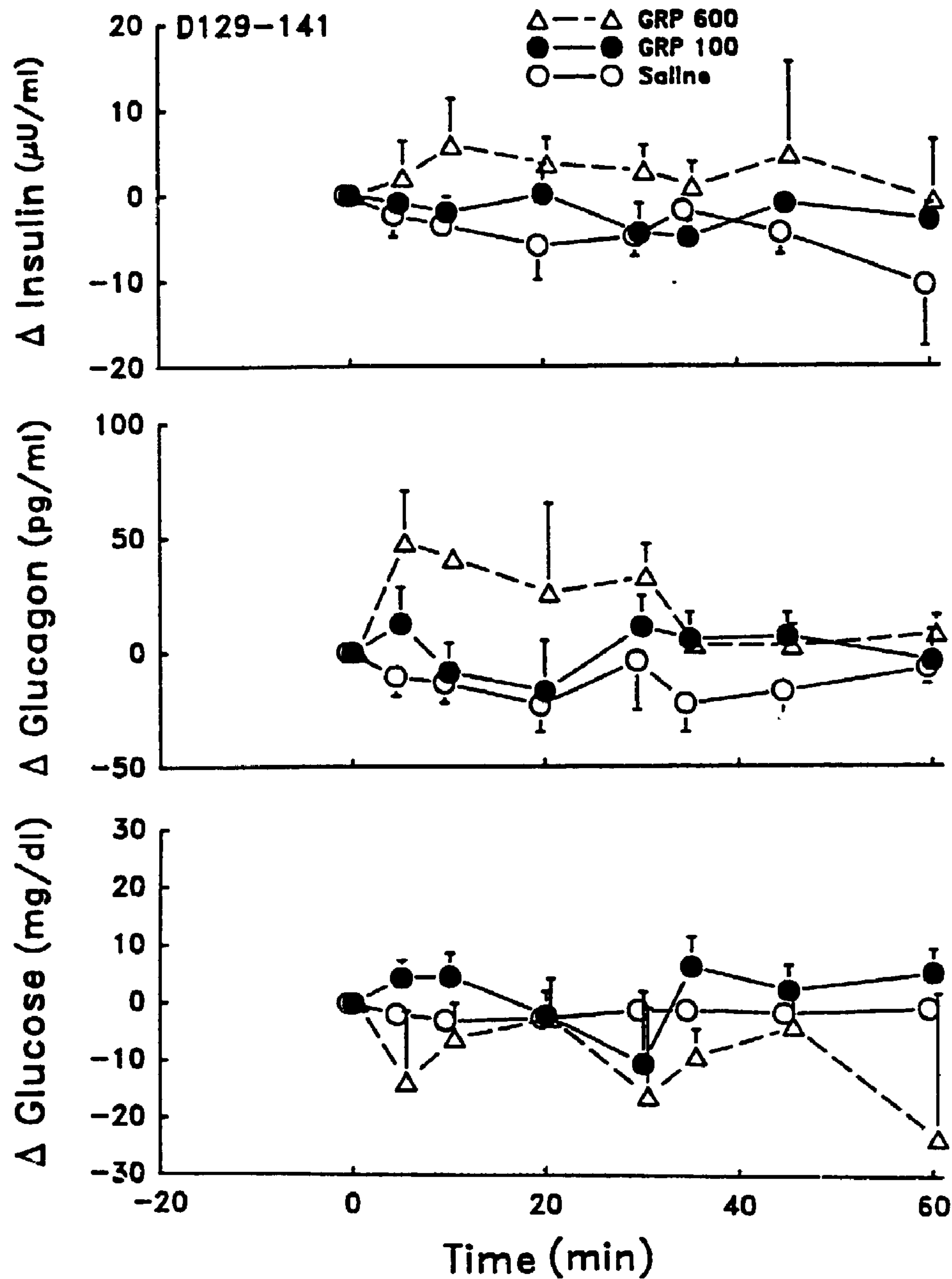

Figure 23. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (O) saline, (●) 100, and (Δ) 600 pmol/kg·hr of GRP into four pregnant ewes between d129-141 of gestation following overnight fasting. (Where no SEM is snown, error is less than the size of the data point).

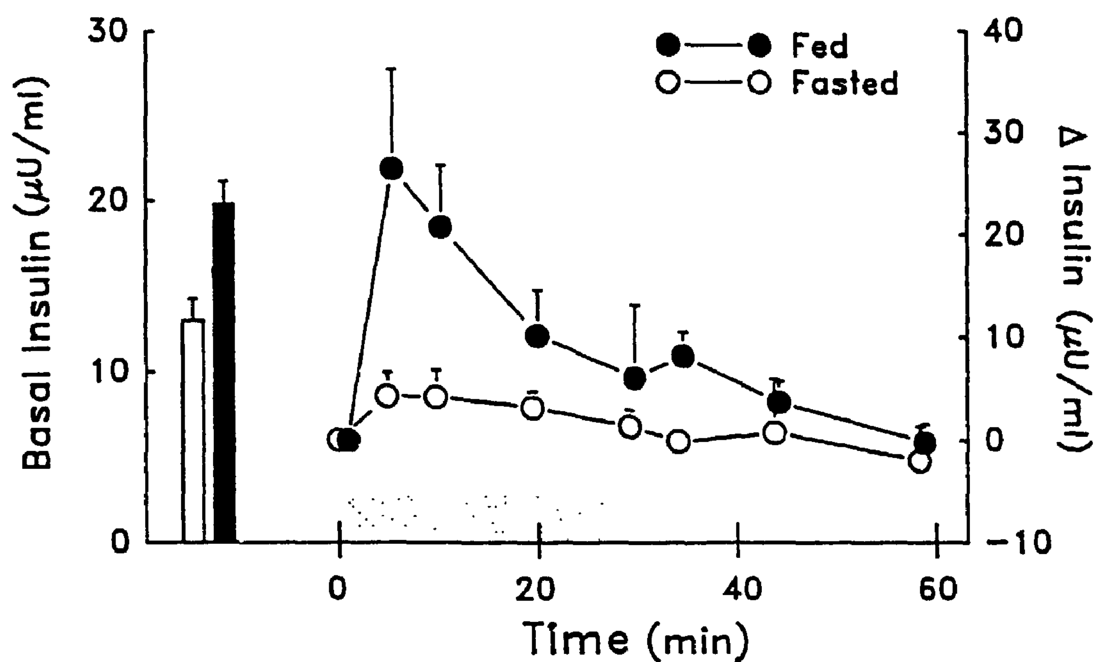

Figure 24. Mean ± SEM of basal insulin concentrations (left histogram) and the change over baseline of plasma insulin concentrations during the infusion of 600 pmol/kg·hr of GRP (right axis) into eight pregnant sheep (●) fed *ad libitum*, and (O) following overnight fasting; n=8. (Where no SEM is shown, error is less than the size of the data point).

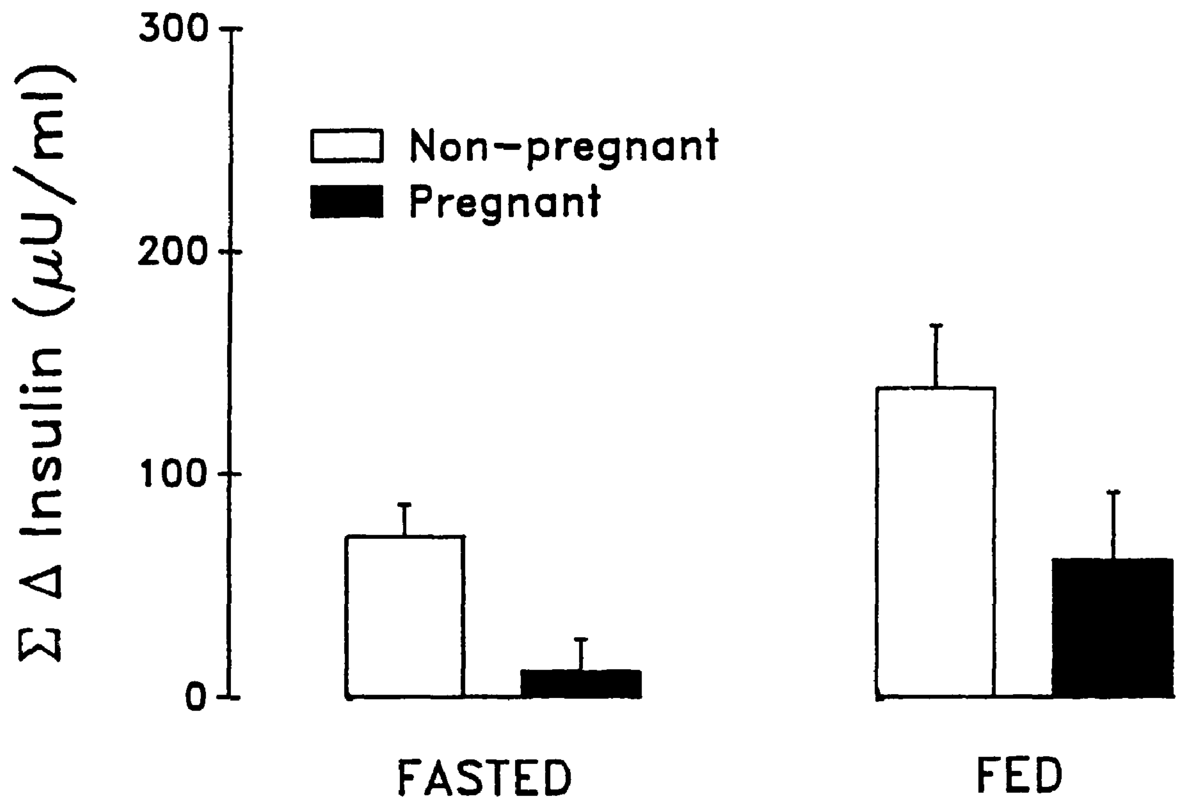

Figure 25. Bar graph of the mean ± SEM of the sum change over baseline of plasma insulin concentrations which occurred following a 30 minute infusion of 600 pmol/kg·hr of GRP into (□) five non pregnant and (■) eight d117-141 pregnant sheep that were (FED) fed *ad libitum*, and (FASTED) fasted overnight.

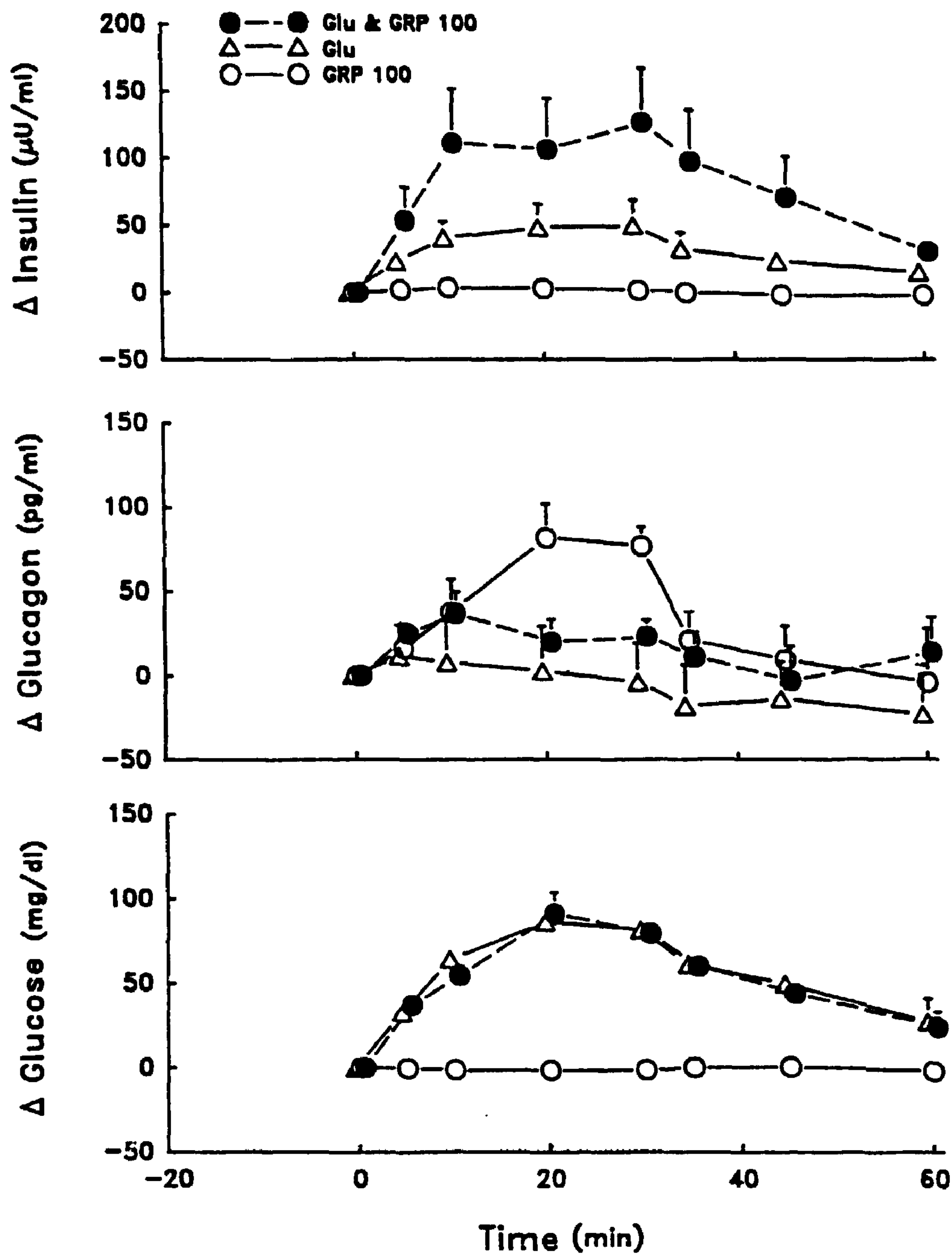

Figure 26. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (O) 100 pmol/kg·hr of GRP given alone, (Δ) 10 mg/kg.min of glucose given alone, and (●) 100 pmol/kg·hr of GRP plus 10 mg/kg.min of glucose given together to five non pregnant ovariectomized sheep that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

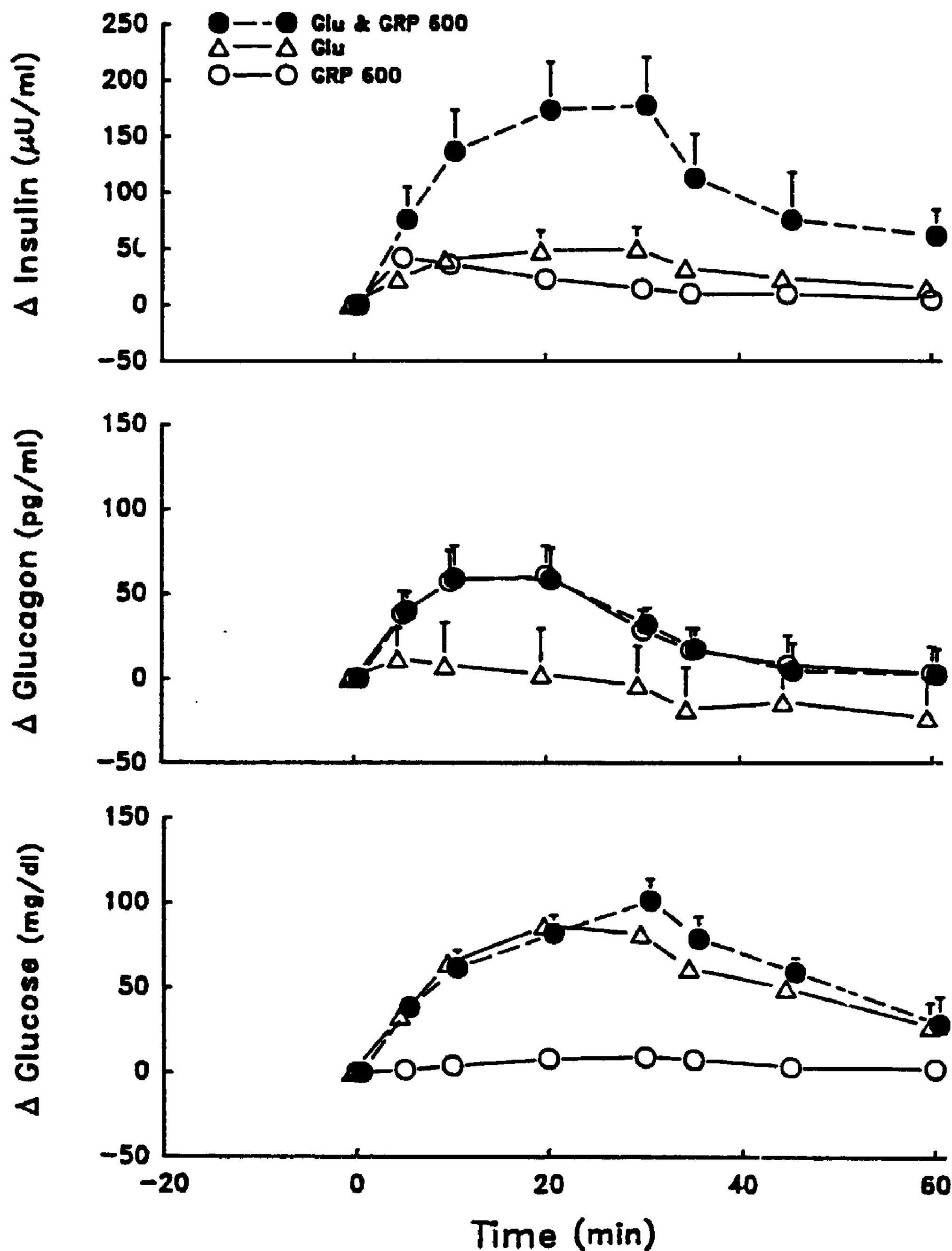

Figure 27. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (○) 600 pmol/kg·hr of GRP given alone, (△) 10 mg/kg.min of glucose given alone, and (●) 600 pmol/kg·hr of GRP plus 10 mg/kg.min of glucose given together to five non pregnant ovariectomized sheep that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

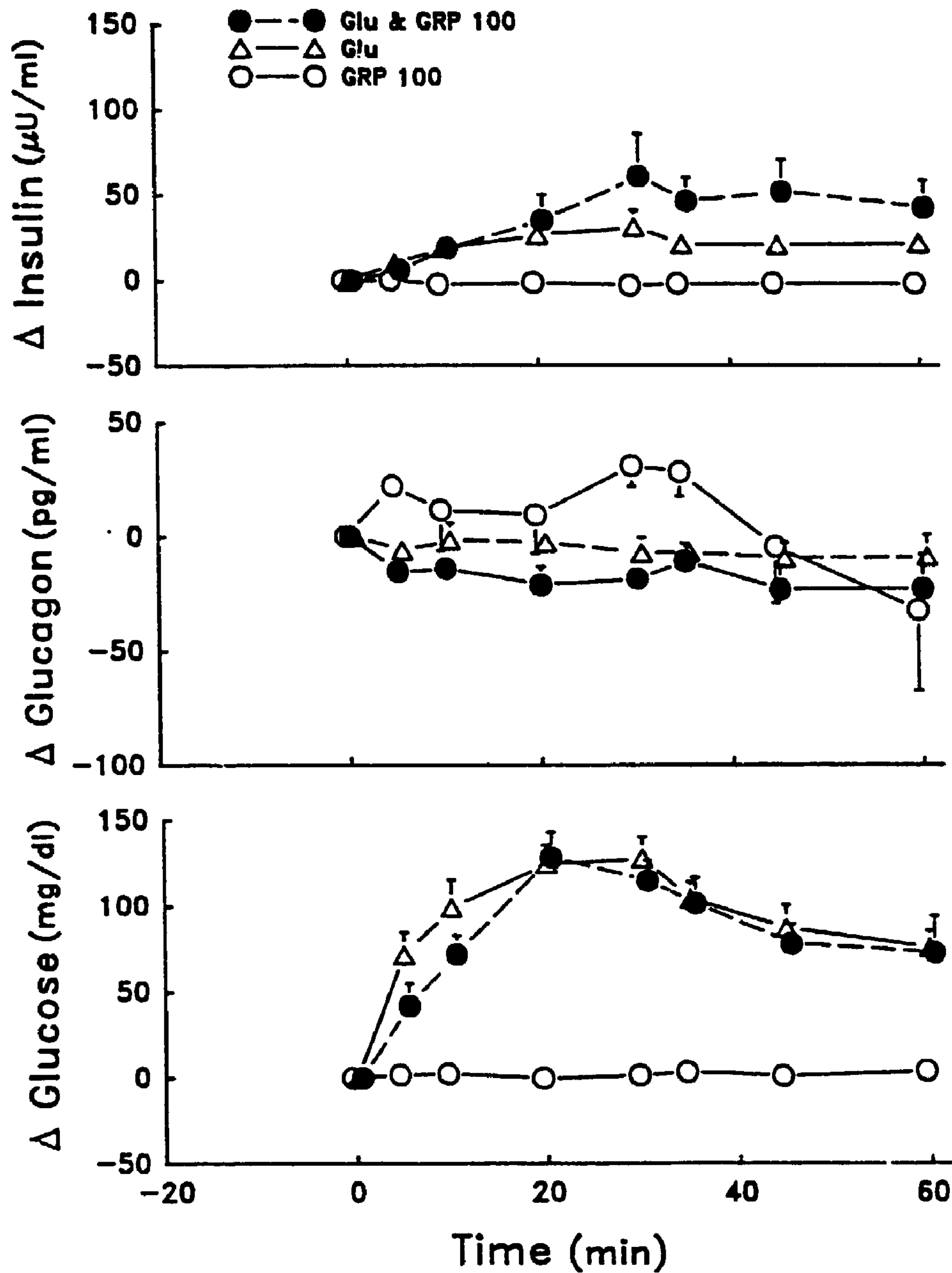

Figure 28. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (○) 100 pmol/kg·hr of GRP given alone, (△) 10 mg/kg.min of glucose given alone, and (●) 100 pmol/kg·hr of GRP plus 10 mg/kg.min of glucose given together to eight d117-141 pregnant ewes that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

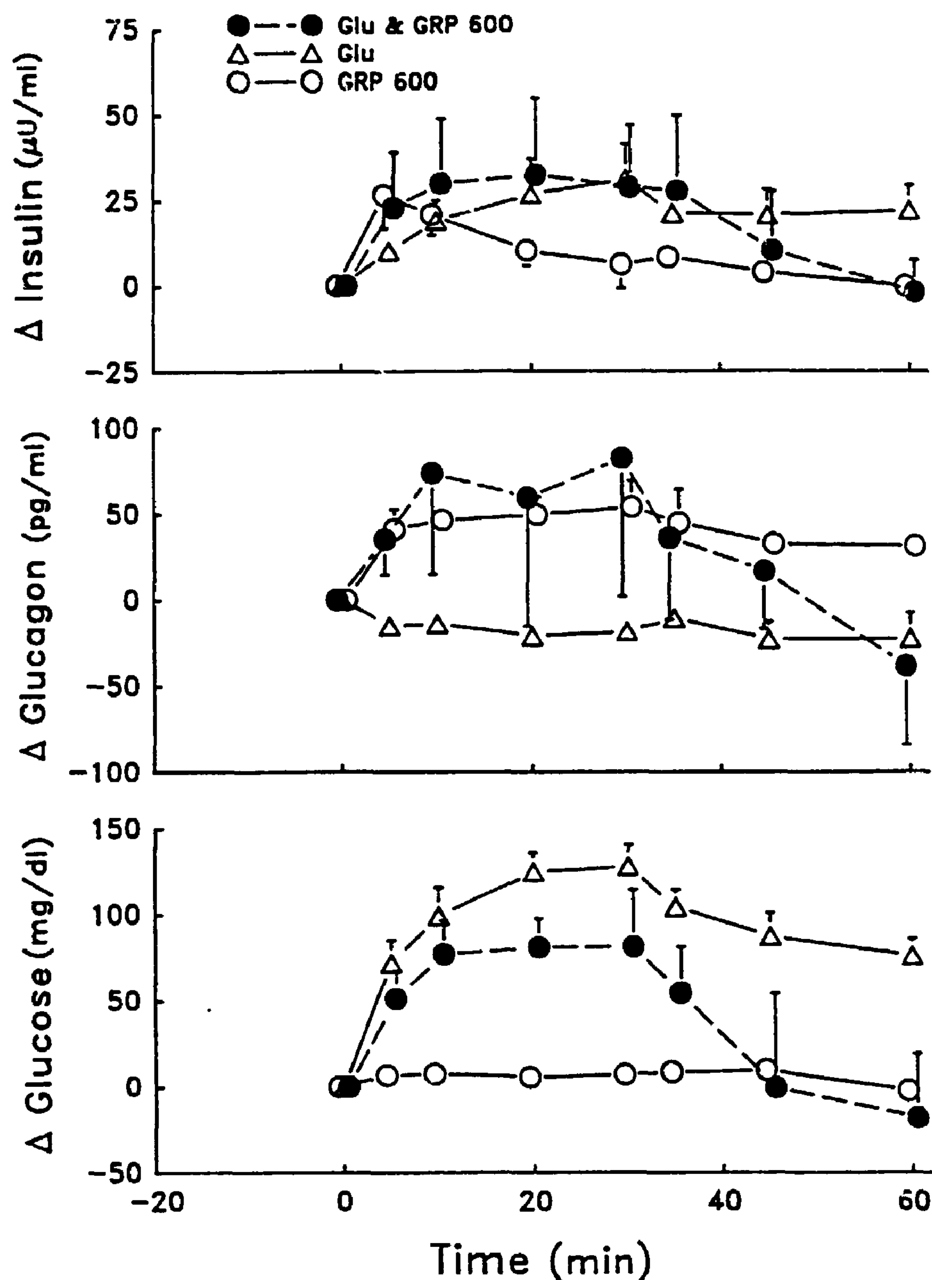

Figure 29. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (O , n=8) 600 pmol/kg hr of GRP given alone, (Δ , n=4) 10 mg/kg.min of glucose given alone, n=8; and (● , n=8) 600 pg/kg/hr of GRP plus 10 mg/kg.min of glucose given together to d117-141 pregnant ewes that were fed *ad libitum*. (Where no SEM is shown, error is less than the size of the data point).

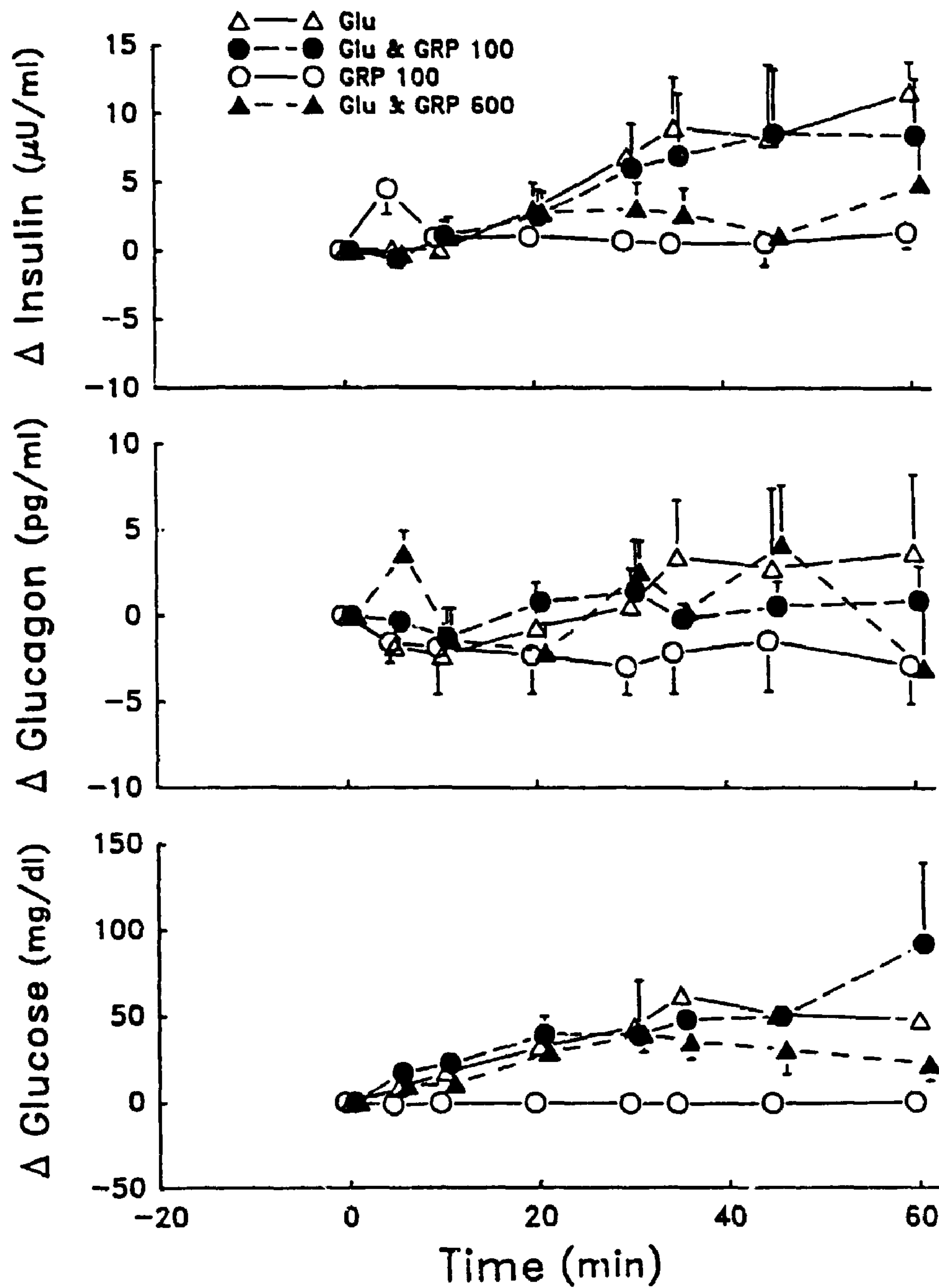

Figure 30. Mean ± SEM of the change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations measured in three fetal sheep following a 30 minute infusion of (○) 100 pmol/kg·hr of GRP given alone, (△) 10 mg/kg.min of glucose given alone, (●) 100 pmol/kg·hr of GRP plus 10 mg/kg.min of glucose administered to together, and (▲) 600 pmol/kg·hr of GRP plus 10 mg/kg.min of glucose administered to together to pregnant ewes fed *ad libitum*.

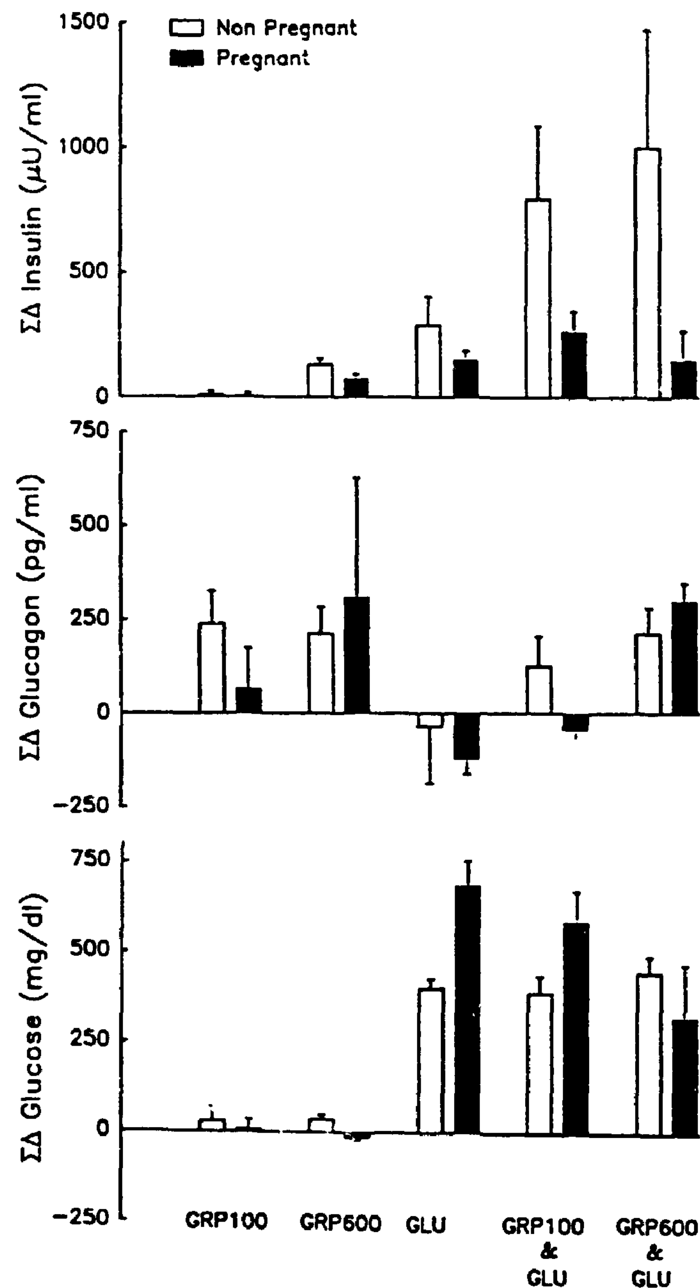

Figure 31. Bar graph of the mean ± SEM of the change over baseline of (top panel) plasma insulin, (midd'e panel) plasma glucagon, (bottom panel) plasma glucose concentrations which occurred in (□) non pregnant sheep, n=5; and (■) pregnant sheep, n=8 except GRP600 & GLU where n=4; following a 30 minute infusion of (GRP100) 100 pmol/kg·hr of GRP given alone, (GRP600) 600 pmol/kg hr of GRP given alone, (GLU) 10 mg/kg.min glucose given alone, (GRP100 & GLU) 100 pmol/kg hr of GRP and 10 mg/kg.min of glucose given together, and (GRP600 & GLU) 600 pmol/kg hr of GRP and 10 mg/kg.min of glucose given together.

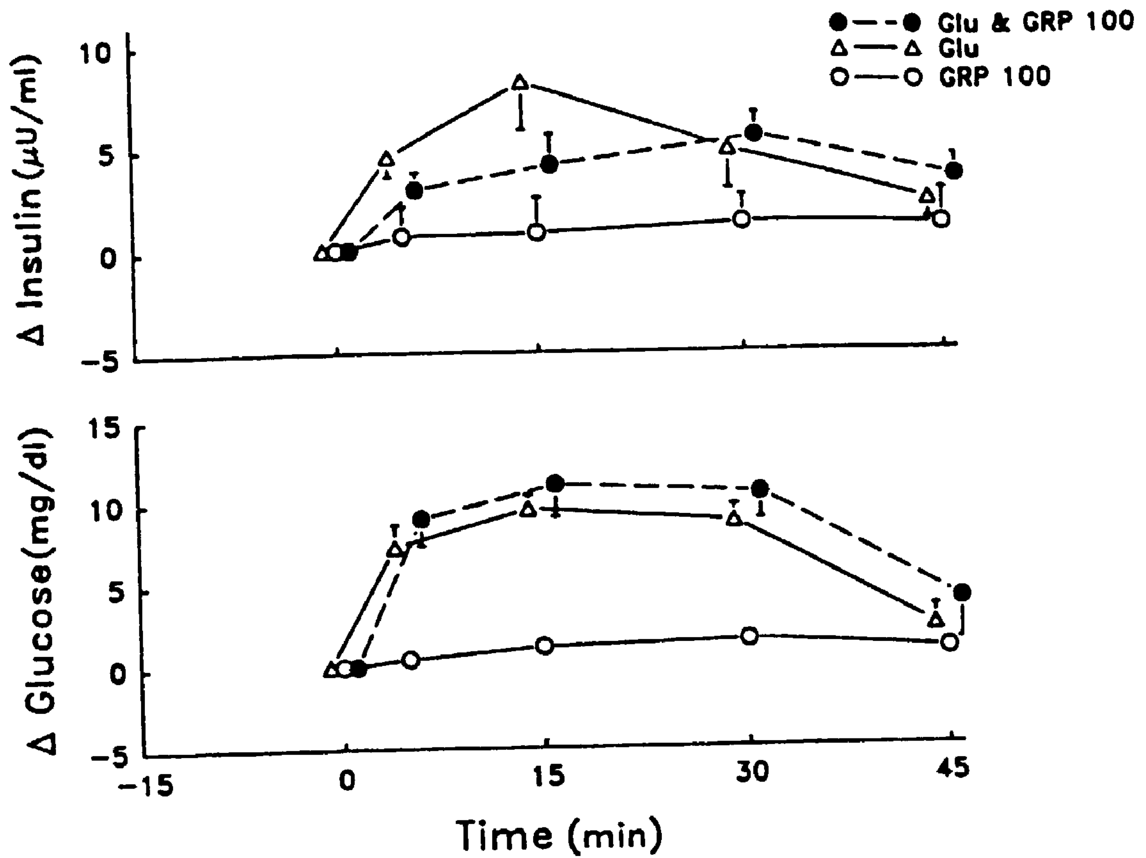

Figure 32. Mean ± SEM of the change over baseline of (top panel) plasma insulin, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (○ , n=11) 100 pmol/kg hr of GRP given alone, (△ ,n=11) 10 mg/kg.min of glucose given alone, and (● , n=11) 100 pmol/kg hr of GRP plus 10 mg/kg.min of glucose given together to fetal sheep between d120 and d140 of gestation. (Where no SEM is shown, error is less than the size of the data point).

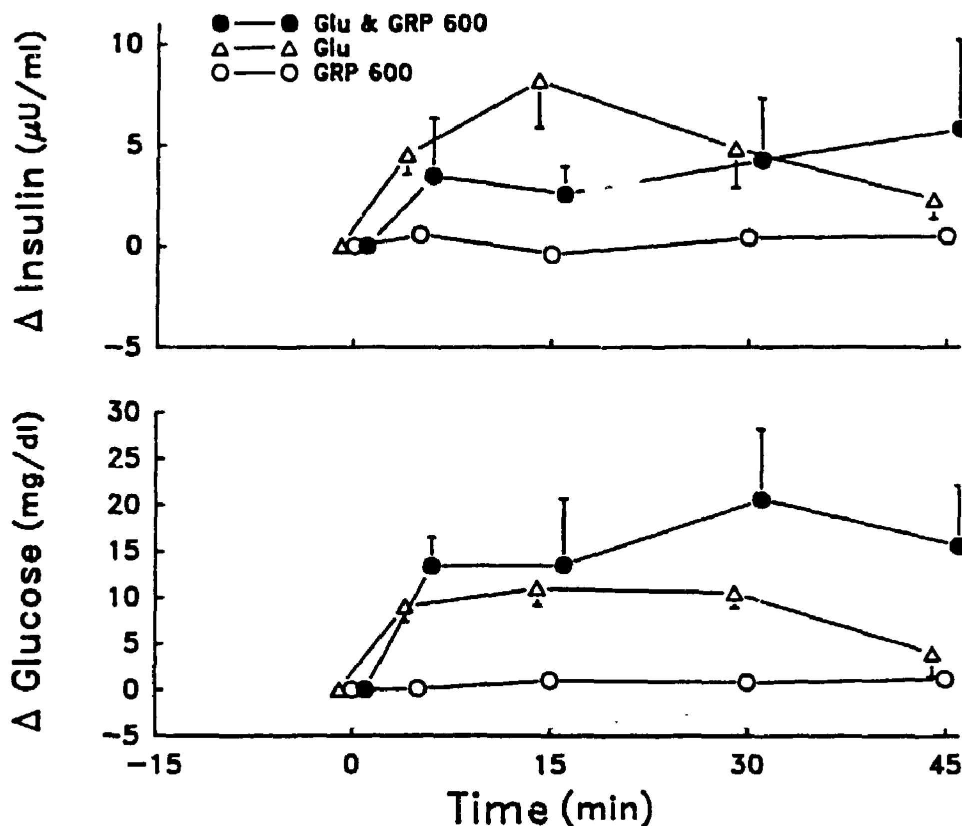

Figure 33. Mean ± SEM of the change over baseline of (top panel) plasma insulin, and (bottom panel) plasma glucose concentrations following a 30 minute infusion of (O , n=8) 600 pmol/kg·hr of GRP given alone, (Δ ,n=11) 10 mg/kg.min of glucose given alone, and (● , n=3) 600 pmol/kg·hr of GRP plus 10 mg/kg.min of glucose given together to fetal sheep between d120 and d140 of gestation. (Where no SEM is shown, error is less than the size of the data point).

8. INSULIN RESPONSE IN ADULT NON-PREGNANT SHEEP: II. MODULATION BY PROGESTERONE (P_4) AND ESTROGEN (E_2)

8.1. INTRODUCTION

In the previous chapter, it was demonstrated that the insulin response to GRP is glucose dependent in adult non-pregnant and pregnant sheep and that the ability for GRP to potentiate glucose-stimulated insulin release is present in non-pregnant but not pregnant sheep (chapter 7). A reduction in the action of potentiators of insulin release such as GRP, may at least in part, be responsible for the impaired glucose tolerance which is known to occur during pregnancy. Very little is known about the effects of pregnancy on the enteroinsular axis. It is known that basal concentrations, and meal-stimulated release of various GI peptides, including GIP, are reduced during pregnancy (Jenssen et al., 1988). Furthermore, Hornnes and co-workers (1984) have demonstrated the impaired incretin effect of GIP is more pronounced in gestational diabetics compared to normal pregnancies. Whether or not GI peptides play a role in the pathophysiology of gestational diabetes is not known. It is important therefore to determine the mechanisms underlying pregnancy-induced alterations in the insulinotrophic actions of various GI peptides that participate in the enteroinsular axis including GRP.

P_4 and/or E_2 have been postulated to be responsible for many of the alterations of metabolism which occur during pregnancy (Bender and Chickering, 1985; Aerts et al., 1980; Baird, 1986). Whether or not P_4 and/or E_2 influence the action of various incretin candidates, such as GRP, is not known. The present experiments examined the hypothesis that P_4 and/or E_2 may, at least in part, be responsible for the reduced insulinotrophic actions of GRP observed in pregnant compared to non-pregnant sheep. To test this hypothesis we examined if the attenuated insulin response to GRP observed in pregnant sheep could be reproduced in non-pregnant ovariectomized sheep treated with P_4 and/or E_2 in amounts that mimic steroid production rates during pregnancy.

8.2. MATERIALS AND METHODS

8.2.1. Animals

Studies were performed on nine adult non-pregnant sheep in which catheters were implanted into the femoral artery and vein as described previously (section 3.4). In addition, the ovarian blood vessels were ligated bilaterally and both ovaries were removed through a small abdominal incision.

8.2.2. Experimental Protocol

Following 7 days post-operative care (provided as described in section 3.3), each of the animals received the following treatments: (1) no steroid treatment (NTX) in which the animals received daily subcutaneous injections of 2.5 ml of corn oil, (2) estradiol treatment (E_2) in which the sheep received daily subcutaneous injections of 25 μg estradiol (Steraloids Inc. Wilton, NH.) dissolved in 2.5ml of corn oil or (3) estradiol plus progesterone treatment (P_4 & E_2) in animals received daily subcutaneous administration of E_2 (25 μg) and P_4 (50 mg; Steraloids Inc. Wilton, NH.) dissolved in corn oil. Steroid treatments were initiated at least 4 days before beginning the experiments and were administered in the order listed (refer to Fig. 34). In five sheep, E_2 was administered for only four days and experiments were not performed in the animals during this time. Therefore these animals had experiments performed while receiving NTX or P_4 & E_2 treatment.

In each of the treatment groups the following test substances were prepared and administered as described previously (section 7.2.3): GRP100, GRP600, Glu, Glu & GRP100, and Glu & GRP600. Test substances were administered at a rate of 2ml/min for 30 min. via the femoral venous catheter, in a randomized order, on separate days between 1200 hr and 1600 hr. Animals were fed twice daily (0800 and 1500 hr) as previously described (section 6.2.1).

Blood samples were collected and processed as described (section 3.4) at -15, -1, +5, +10, +20, +30, +35, +45, and +60 min. relative to the onset of the infusion. Plasma glucose, insulin, and glucagon concentrations were determined as described previously (section 3.5 & 3.6).

8.2.3. Circulating Progesterone (P_4) and Estradiol (E_2) Concentrations

A preliminary study was conducted in order to establish the time following the bolus subcutaneous injection of steroids in which circulating steroid concentrations were similar to those present in pregnant sheep during d117-141 of gestation. In this study, arterial plasma from four non-pregnant ovariectomized sheep was collected before and at intervals of 0.5 to 2.0 hr for 36 hr after daily subcutaneous injection of E_2 and P_4. Plasma P_4 and E_2 concentrations were determined as described previously (section 3.4) and compared to circulating concentrations measured in pregnant sheep during d117-141 of gestation.

8.2.4. Data Analysis

Results are presented as the sum of the changes in plasma insulin, glucagon, and glucose concentrations in adult sheep during GRP and/or glucose administration in the three different treatment groups (NTX, E_2, and P_4 & E_2). Results in pregnant sheep have been described in detail in the previous chapter and are included here for the purpose of comparison. Since no significant differences were demonstrable between any of the responses observed in the pregnant sheep at different gestational ages, the results have been pooled. Sum changes in plasma concentrations were calculated as the mean ± SEM of the summed increases over the average of the two pre-injection values for each individual animal. One way analysis of variance was used to test for significant differences between non-pregnant sheep in different treatment groups and between non-pregnant and pregnant sheep. Basal glucose, insulin, glucagon, P_4, and E_2 concentrations

(presented in Table 3) were determined using the mean of the two pre-injection values for each of the individual animals in the groups indicated and were compared statistically using a one way analysis of variance. Differences were considered statistically significant at $p<0.05$.

8.3. RESULTS

8.3.1. Basal Concentrations of Plasma Glucose, Insulin, Glucagon and Steroids

The concentration of E_2 in plasma rose from less than 5-25 pg/ml to greater than 75 pg/ml during the 2 hr after the injection of E_2, and then decreased over the next 6 hr to values of 10-20 pg/ml at 8-24 hr after the injection (Fig. 35). The plasma concentration of P_4 rose to approximately 25-30 ng/ml immediately after the subcutaneous steroid injection and then decreased over the next 2 hr to ≈ 10-15 pg/ml where they remained relatively constant over the next 4-6 hr (Fig. 35). Based on these results, experiments were conducted in non-pregnant ovariectomized sheep 4 - 8 hr after a bolus injection of corn oil with or without P_4 and/or E_2.

Mean basal P_4 and E_2 concentration measured in d117-141 pregnant sheep in samples taken immediately before infusion of the test substances were 22 ± 3 pg/ml and 14 ± 3 ng/ml, respectively. Mean basal E_2 concentrations in non-pregnant sheep ovariectomized sheep treated with steroids were 42 + 6 pg/ml which was similar but significantly greater than basal E_2 concentrations measured in d117-141 pregnant ewes (22 ± 3 pg/ml; Table 3; $p<0.05$). However, mean basal P_4 concentration were not significantly different between pregnant (14 ± 3 ng/ml) and non-pregnant sheep treated with steroids (14 ± 2 ng/ml).

Basal concentrations of plasma glucose, insulin, were not significantly different between non-pregnant sheep with or without steroid treatment ($p<0.05$). Basal glucagon concentrations measured in the plasma of non-pregnant sheep not treated with steroids were significantly greater than the same animals receiving steroid treatment (Table 3; $p<0.05$).

8.3.2. Comparisons between Non-pregnant Sheep with and without Steroid Treatment and Pregnant Sheep

Results obtained from experiments in non-pregnant sheep treated with P_4 & E_2 were not significantly different between those animals in which previous experiments were performed during E_2 treatment and animals that had received only E_2 priming for 4 days. As a result of these similarities, the results in the two groups of P_4 & E_2 treated non-pregnant sheep were pooled.

a) Changes in Plasma Glucose Concentrations

A similar dose of glucose produced significantly greater elevations in plasma glucose concentrations in pregnant (689 ± 70 mg/dl) compared to non-pregnant sheep ovariectomized sheep without (403 ± 27 mg/dl; $p<0.05$) and with P_4 & E_2 treatment (396 ± 79 mg/dl; $p<0.05$) but not significantly different than the same animals treated with E_2 alone (545 ± 20 mg/dl; refer to Fig. 36). The changes in plasma glucose concentrations that occurred following administration of GRP600 and Glu & GRP100 also exhibited similar trends in pregnant and non-pregnant ovariectomized sheep treated with E_2 alone. However, these trends were not statistically significant.

b) Insulin Responses

The insulin response (expressed as the sum change over baseline of plasma insulin concentrations) to the infusion of GRP600 was significantly greater in non-pregnant ovariectomized sheep (139 ± 28 µU/ml; $p<0.05$) compared to pregnant sheep (58 ± 8

μU/ml; $p<0.05$) and non-pregnant animals receiving P_4 & E_2 treatment (72 ± 16 μU/ml; $p<0.05$) but not E_2 treatment (309 ± 100 μU/ml; Fig. 36). The insulin response to GRP100 alone and Glu alone was not significantly different between treatment groups, however, the sum change in plasma insulin concentrations following Glu & GRP100 administration were statistically less in P_4 & E_2 treated non-pregnant sheep (246 ± 56 μU/ml) and pregnant sheep (261 ± 86 μU/ml) compared to those measured in non-pregnant ovariectomized sheep without steroid treatment (595 ± 187 μU/ml; Fig. 36; $p<0.05$). However, the insulin response to Glu & GRP100 was not significantly different between non-pregnant sheep treated with corn oil and those treated with E_2 alone. Similar trends were observed in pregnant and non-pregnant sheep with and without steroids during the infusion of the higher dose of GRP (GRP600) given separately or together with glucose. However, due to large variations in absolute values these differences were not statistically significant (Fig. 36). Treatment of non-pregnant ovariectomized animals with E_2 resulted in a significantly greater sum change in plasma insulin concentrations in response to either dose of GRP given alone, compared to any of the other treatment groups (Fig. 36; $p<0.05$).

b) Glucagon Responses

GRP administration tended to increase plasma glucagon levels whereas plasma glucagon concentrations tended to decrease following glucose administration (Fig. 36). These opposing effects were cancelled when Glu and GRP100 were administered together but the stimulatory effect of GRP predominated when the higher dose of GRP (GRP600) was given together with glucose. However, there were no statistically significant differences in the glucagon responses between pregnant and non pregnant sheep with or without steroid treatment.

8.4. DISCUSSION

In these studies, we have demonstrated that both the ability of GRP to stimulate insulin release and to potentiate glucose-stimulated insulin release present in adult non-pregnant ovariectomized sheep is abolished by exogenous administration of physiological amounts of both P_4 and E_2 but not E_2 alone. These results suggest that reduced action of GRP demonstrated previously in pregnant sheep, is mediated, at least in part, by the high circulating concentrations of P_4 which are present in late gestation. In addition, these results suggest that is unlikely that the reduced actions of GRP observed in pregnant sheep are due to either the dilution of GRP within a greater blood volume or altered GRP metabolism which may occur in pregnant compared to non-pregnant sheep.

Both E_2 and P_4 concentrations are elevated in late gestation pregnant sheep (Challis and Olson, 1988). E_2 is known to be necessary to induce and sustain the P_4 receptor in target tissues (Vu Hai et al., 1977; Leavitt et al., 1977). Therefore it was necessary to examine the effects of P_4 in non-pregnant ovariectomized sheep which had received previous and concurrent E_2 administration. However, similar results were obtained from the first series of experiments performed in P_4 and E_2 treated non-pregnant sheep which only had previous experiments performed during NTX treatment compared to those obtained in a second series of animals in which previously experiments had been conducted during not only NTX treatment but also during E_2 treatment. It is unlikely therefore that time after surgery or the effects of previous experiments during E_2 treatment were responsible for the observed differences following P_4 & E_2 treatment. As our experimental model precluded the administration of steroid treatment in a randomized order, we cannot rule out the possibility that previous experiments in the NTX treated sheep did not produce the observed differences. Also, because P_4 was not administered alone, it is possible that the observed effects were due to the synergistic effects of the two

steroids which would not be observed when the two steroids were administered alone. This may indeed occur since administration of E_2 alone did significantly increase the insulin reponse to GRP alone when compared to the responses in the same animals without steroid treatment. This injection regime in no way duplicates the gradual rise in plasma steroid concentrations which occur during pregnancy. Bolus subcutaneous injections produced daily ten fold fluctuations in steroid concentrations. However, it does produce circulating P_4 levels at the time of the experiments were carried out which were not statistically different from plasma P_4 levels measured in pregnant sheep during late gestation. Basal E_2 concentrations in samples taken immediately before the experiments were however significantly greater than circulating E_2 concentrations measured in pregnant sheep. Despite these shortcomings, this experimental model does have the distinct advantage of allowing us to examine the effect of manipulating P_4 and/or E_2 concentrations while keeping other potential agents relatively constant. This is critical when studying factors underlying pregnancy associated changes since pregnancy is associated with such marked changes in virtually every physiological system. These results do not eliminate the possibility that other changes which occur during pregnancy may also act to decrease the insulinotrophic actions of GRP observed during pregnancy. PL is secreted by the placenta and in increasing amounts throughout gestation (Josimovich et al., 1970). Studies have demonstrated that PL administration results in impaired glucose tolerance (Beck and Daughaday, 1967; and Grumbach et al., 1968) while others have found no consistent effect (Beck, 1970). *In vitro* studies reveal that PL stimulates DNA synthesis and increased insulin release in rat and mouse islets (Freinkel, 1980). Therefore these *in vitro* studies suggest that PL is capable of altering β-cell function directly while *in vivo* studies reveal conflicting results. Other pregnancy associated changes include increased blood volume and alterations in the metabolism of

many different humoral factors. The results obtained using this experimental model has allowed us to eliminate the possibility that attenuated effects of GRP observed during pregnancy are solely due to either dilution of GRP within the greater blood volume or an accelerated rate of GRP clearance and strongly suggest that P_4 is an active mediator of this response.

The mechanism by which P_4 exerts its effects is unknown. Pregnancy associated ultrastructural changes in the endocrine pancreas such as increases in the number of light granules and a distinct swelling of the β-cell mitochondria can be reproduced by the direct application of P_4 to *in vitro* pancreatic cultures (Aerts et al., 1980). Further, P_4 & E_2 receptors have been localized in the pancreatic islets (Green et al., 1981). Therefore these steroids appear to be able to alter pancreatic endocrine secretion by acting directly on the islets presumably via alterations in gene expression.

In summary, these studies suggest that changes in carbohydrate metabolism including the reduced insulinotrophic action of GI peptides such as GRP, may be due to the direct effects of P_4 on the pancreatic islets and that P_4 may mediate the reduced ability for GRP to stimulate insulin release and potentiate glucose-stimulated insulin secretion which occurs in pregnant sheep.

Treatments: 1. Saline
2. GRP100
3. GRP600
4. Glu
5. GRP100 & Glu
6. GRP600 & Glu

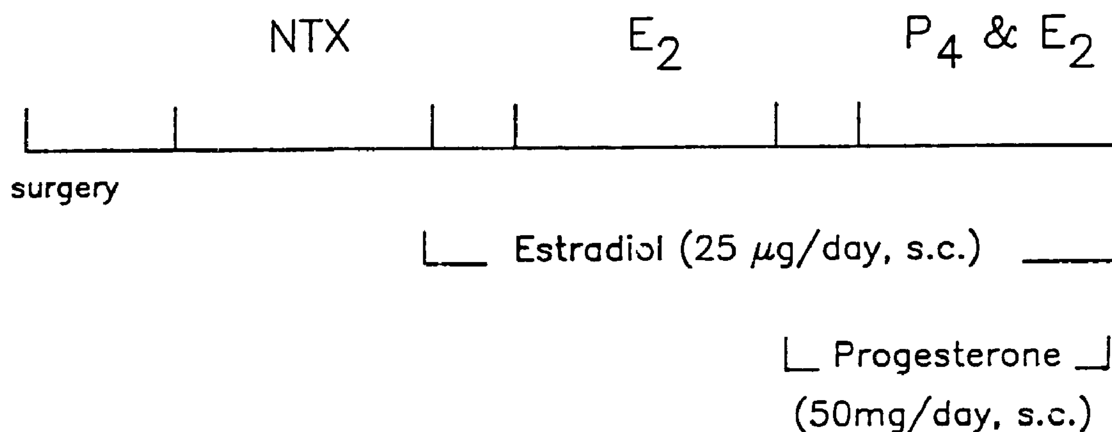

Figure 34. Diagramatic representation of the injection regime for nine non pregnant ovariectomized sheep over time. Treatments 1 - 6 were administered on separate days, at the same time of day, in a randomized order to animals which received daily subcutaneous injections of (NTX) 2.5 ml corn oil, (P4E2) both progesterone (50 mg) and estradiol (25 µg) dissolved in 2.5 ml corn oil, and (E2) estradiol (25 µg) alone dissolved in 2.5 ml corn oil for 4 days before and during treatments.

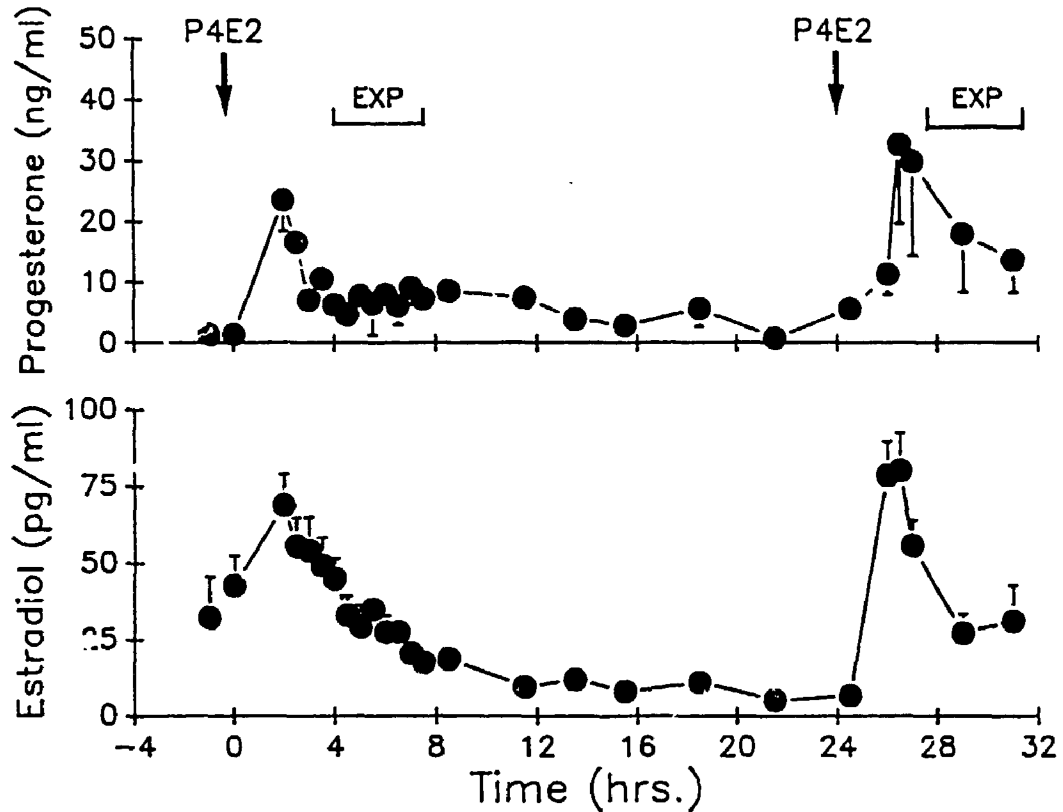

Figure 35. Mean ± SEM of the changes in plasma progesterone and estrogen concentrations which occurred in four non pregnant sheep over 36 hours of sampling. Animals were fed at 0 and 8 hours but not at any time point thereafter. Progesterone (50 mg) and estradiol (25 μg) dissolved in corn oil were administered as a daily subcutaneous injection at 0 hours (as indicated; P4E2 arrows) and experiments were carried out between 4 and 8 hours thereafter (as indicated; EXP). (Where no SEM is shown, error is less than the size of the data point).

	Glucose (mg/dl)	Insulin (μU/ml)	Glucagon (pg/ml)	Prog. (ng/ml)	Estradiol (pg/ml)
PREGNANT SHEEP	72 ± 3	17 ± 2	76 ± 11	14 ± 2	22 ± 3
NON–PREGNANT SHEEP					
No Treatment	60 ± 1	12 ± 1	112 ± 8	ND	ND
P4 & E2 Treatment	61 ± 1	8 ± 1	82 ± 7	14 ± 3	42 ± 6
E2 Treatment	67 ± 2	14 ± 2	71 ± 9	----	----

Table 3. Mean ± SEM of plasma insulin, glucose, glucagon, progesterone, and estradiol concentrations measured in preinjection samples collected from d117-141 pregnant sheep and non pregnant ovariectomized sheep not treated with steroids and the same animals treated with daily subcutaneous injections of both 50 mg of progesterone and 25 μg of estradiol together and 25 μg of estradiol alone. 'ND'= not detectable, '----' = not measured.

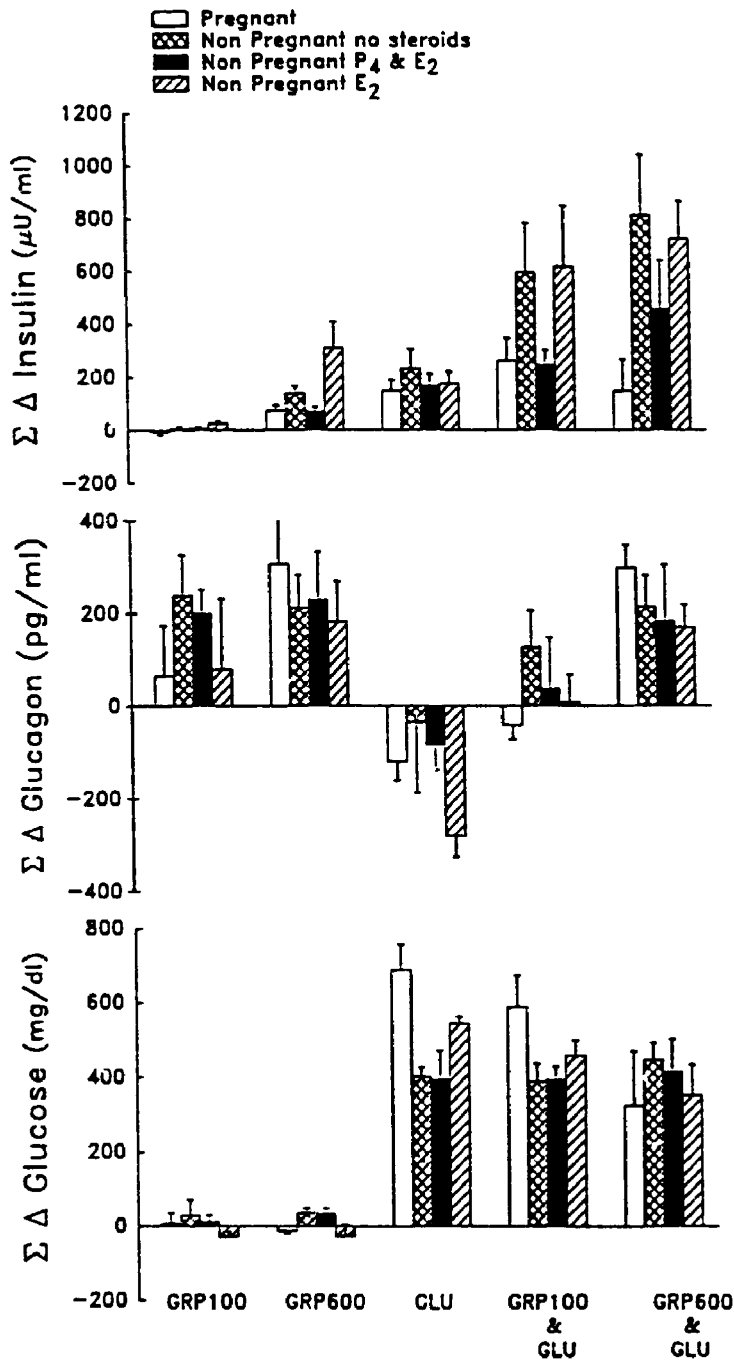

Figure 36. Bar graph of the mean ± SEM of the sum change over baseline of (top panel) plasma insulin, (middle panel) plasma glucagon, and (bottom panel) plasma glucose concentrations which occurred following a 30 minute infusion of (GRP100) 100 pmol/kg hr of GRP given alone, (GRP600) 600 pmol/kg hr of GRP given alone, (GLU) 10 mg/kg.min of glucose given alone, (GRP100 & GLU) 100 pmol/kg hr of GRP plus 10 mg/kg.min of glucose given together, and (GRP600 & GLU) 600 pmol/kg hr of GRP plus 10 mg/kg.min of glucose given together to (□ ; n=8) pregnant sheep and non pregnant sheep (▩ ; n=9) with no steroid treatment, given daily subcutaneous injections of (■ ; n=9) both progesterone (50 mg) and estradiol (25 μg), and (▨ ; n=4) estradiol (25 μg) alone.

9. GRP MEASUREMENTS

9.1. INTRODUCTION

Studies performed in different monogastric species have led to the suggestion that GRP is released locally from neural structures onto the β-cell to stimulate insulin release (Knuhsten et al., 1984; Ghatei et al., 1984). As GRP is a neuropeptide, its presence is not necessarily detectable in the systemic circulation and the lack of change in plasma GRP concentrations does not preclude GRP from being an important component of the enteroinsular axis. Definitive evidence for GRP being a physiological regulator of insulin release in monogastric species probably awaits the development a potent and specific GRP antagonist.

Bloom and co-workers (1983b) have demonstrated that GRP administered intravenously to conscious calves produces dose dependent elevations in insulin levels and that the ability of GRP to stimulate insulin release in these ruminants was dependent on circulating glucose and amino acid concentrations (Bloom et al., 1984). These results are consistent with previous reports in monogastric animals (Greeley and Thompson, 1984; Martindale et al., 1984; Wood et al., 1983). Furthermore, previous results presented in this thesis (Chapter 7) have demonstrated that GRP potentiates glucose-stimulated insulin release in non-pregnant sheep. Immunocytochemical studies have revealed GRP-containing nerves are undetectable in the pancreas of calves and goats (Bloom et al., 1983b; Dockray et al., 1979). Hence, an anatomical basis for GRP acting via local release onto the pancreatic islets of ruminants has not been documented. However, GRP is detectable in the systemic circulation of conscious calves (Bloom et al., 1983a; Bloom and Edwards, 1984) and it is therefore possible that GRP may act in an endocrine fashion to exert insulinotropic effects. Further species differences between ruminant and non-ruminants exist as elevations in circulating GRP concentrations in calves were produced

by splanchnic (Bloom et al., 1980) but not by vagal (Adrian et al., 1983) nerve stimulation which increased GRP concentrations in the pancreatic venous effluent of an isolated perifused pancreas of pigs (Knuhsten et al., 1984). Bloom and his colleagues (1984) have also suggested, based on a preliminary study, that feeding of calves produced significant increases in circulating plasma BLI. If completed studies confirm these preliminary reports, they would strongly support a possible physiological role for GRP as a neuroendocrine agent capable of stimulating the pancreas of ruminants. Whether or not this is the mode by which GRP exerts its insulinotropic effects in sheep remains to be determined.

The purpose of the following series of experiments was to determine whether GRP exerts its insulinotropic effects in adult sheep by acting systemically and attempt to explain the lack of an effect of GRP administration on plasma insulin concentrations in fetal sheep.

The following specific questions were addressed: (1) What is the distribution and localization of GRP within the GI tract and pancreas of fetal and adult sheep? (2) Is GRP detectable in the systemic circulation of fetal or adult sheep? (3) Can alterations in plasma GRP concentrations be detected following feeding of adult sheep?

9.2. METHODS

9.2.1. Tissue Extraction

Portions of pancreas were rapidly excised from a fetal and an adult non-pregnant sheep, sacrificed by administering an overdose of euthanyl (M.T.C. Pharmaceuticals, Miss, Ont.). GRP content was determined in pancreatic tissues using the following extraction technique which has been previously employed to extract GRP from various tissues in many other species (Ghatei et , 1984). Excised tissues were quickly frozen using liquid

nitrogen and then stored at -70°C until subsequent analysis. Pancreatic tissue was immersed in boiling water (9 gm H_2O per 1 gm tissue), and then cooled to 4°C, glacial acetic acid (ACOH) was added to attain a 3% (v/v) concentration, and the samples were extracted overnight on a rotating mixer at 4°C. Samples were centrifuged for 45 min. at 3000 x g at 4°C. The supernatant was removed, centrifuged at 20,000 x g until clear (45 - 60 min.), and then diluted with water to give a final concentration of 0.2 M ACOH. Samples were lyophilized and reconstituted in the buffer used for radioimmunoassay of GRP. This buffer consisted of 0.6M sodium phosphate (dibasic), 0.05% sodium azide, and .01M EDTA with the pH adjusted to 7.55 using 5 M phosphoric acid. GRP concentration was determined in serial dilutions of sample aliquots of the tissue extracts using RIA techniques described in the next section.

9.2.2. GRP Radioimmunoassay (RIA)

Tissue and plasma immunoreactive GRP (IR-GRP) concentrations were measured using a RIA described previously (McDonald et al., 1988b). Plasma samples were assayed using one of two different antiserum (final dilution = 1:30,000) which have been extensively characterized previously; AB103 (Ghatei et al., 1982) and LR-16 (McDonald et al., 1988b). Initially, plasma samples were assayed using the AB103 antiserum since previous studies have shown that this antiserum can detect GRP levels as low as 5 fmol/ml (Ghatei et al., 1982). This antiserum was raised in rabbits against bombesin but can detect GRP and bombesin equipotently. The minimum assay sensitivity (assessed as three standard deviations around the zero binding counts) was 8 ± 4 fmol/ml in the three assays performed in this study using the AB103 antiserum. Intra- and inter-assay coefficients of variation were less than 10%. The AB103 antiserum cross reacts 1-2% with the structurally related tachykinins including substance P, and neuromedin B (McDonald, personal communication) but does not cross react with other structurally

unrelated gastroenteropancreatic (GEP) peptides (Ghatei et al., 1982). Fetal plasma IR-GRP levels were determined using the LR-16 antiserum. This antiserum was raised in rabbits against porcine synthetic GRP (1-27) and has less than a 0.01% cross reaction with tachykinins including substance P and neuromedin B, and has no cross reaction with any structurally unrelated GEP peptides tested (McDonald et al., 1988b). However, in the present studies, the minimum detectable dose using the LR-16 antiserum was 70 fmol/ml. Intra- and inter- assay coefficients of variation were 8.7% and 19% (n=2), respectively. Serial dilutions of fetal sheep plasma produce parallel dose inhibition curves as the synthetic porcine GRP (1-27) standard used in the assay (see Fig. 37).

9.2.3. Immunohistochemical Techniques

Portions of the pancreas from term fetal sheep (n=4), 24-48 hr post-natal lambs (n=5), and adult non-pregnant sheep (n=6) and of the GI tract of d120-141 fetal sheep (n=5) and neonatal sheep (n=3) were obtained following sacrifice. Tissues were placed in Bouin's fixative for 2 hr, and then washed and stored in 70% ethanol. The tissues were then dehydrated through graded alcohols, embedded in paraffin wax at 60°C, cut in 5 µm sections, and placed on slides for staining.

In all cases, sections were incubated with the primary antisera (LR-16; 1:1000) for two days at 4°C. This was followed by a 1 hr incubation at room temperature (RT) with biotinylated-donkey anti-rabbit IgG (1:3000 Jackson Laboratories, PA) and then incubated with tetra-methyl-rhodamine-isothiocyanate-conjugated avidin complex (rhod; 1:400, Vector Laboratories, CA) for l hr at RT. Finally, the slides were washed in phosphate buffered saline (PBS), pH 7.4, and the cover slips applied in PBS/Glycine (1:9).

In control studies, the primary antiserum was preincubated with 10 nM GRP overnight at 4°C. Other control incubations involved the use of normal rabbit serum (NRS) and PBS in place of the primary antiserum.

9.2.4. Plasma GRP Concentrations Produced by Exogenous GRP Administration

Plasma samples were taken from four adult pregnant, and four non-pregnant ovariectomized sheep without steroid treatment and the same animals with daily subcutaneous injections of E_2 given separately and together with P_4 (as described previously in section 8.2). Plasma IP-GRP concentrations were determined in these animals in samples taken between 15 min. before and up to 60 min. after the onset of a 30 min. infusion of either: (1) GRP100, (2) GRP600, (3) Glu, (4) Glu & GRP100 or (5) Glu & GRP600. In addition, plasma IR-GRP concentrations were determined in fetal and maternal plasma samples collected before and after the administration of GRP100, GRP600, Glu & GRP100, and Glu & GRP600 into the fetal venous catheter and in fetuses when GRP and/or glucose was infused into the pregnant ewe.

9.2.5. Endogenous IR-GRP Concentrations In Relation to Feeding and Fasting

Under general anaesthesia four adult non-pregnant sheep had catheters implanted into the femoral artery and vein and both ovaries removed through a small abdominal incision as described previously (section 3.2). Post-operative procedures were carried out for the next 7 days. On the first day following recovery, animals were fed 0.4 kg of grain (see Appendix II for food composition) at 0800 hr, which was consumed within approximately 30 min. This was then followed with a bucket of hay. This same feeding regime was repeated later on the same day (1500 hr). Blood samples were collected at 15 min. intervals for 30 min. before and up to 120 min. after feeding and at 120 min. intervals between these times. The second day of the experiment, the animals received only fresh water at 0800 and 1500 hr which we have termed 'sham feeding'. At 0800 hr on the third day, the animals were fed grain and hay as described and blood samples were collected every 15 min. for 30 min. before and up to 120 min after feeding.

Blood samples were processed as described previously (section 3.4) and plasma glucose, insulin, glucagon, and IR-GRP concentrations were determined using RIA techniques as described previously (section 9.2.2).

9.3. RESULTS

9.3.1. IR-GRP Tissue Content and Localization

Nerves containing IR-GRP were not demonstrable in pancreas of either fetal, neonatal, or adult sheep using immunocytochemical techniques. In confirmation, IR-GRP immunoreactivity in pancreatic extracts of fetal and adult sheep was also undetectable. Positive staining was detected within neural structures of the GI tract of neonatal and fetal lambs. IR-GRP containing nerve fibers were present within the myenteric plexus and circular muscle of the duodenum (see Fig. 38) and abomasum of newborn and fetal sheep.

Preabsorption of the primary antiserum with 10 nM GRP or replacement of the primary antiserum with NRS or PBS abolished the immunostaining in all regions and thus confirmed the specificity of the staining produced by the primary antiserum.

9.3.2. Basal Plasma IR-GRP Concentrations

IR-GRP was present in the systemic circulation of d118 to d135 fetal sheep. Fetal basal GRP concentrations were approximately 100 fold greater than those measured in adult pregnant and non-pregnant sheep ($p<0.05$; Table 4).

IR-GRP was also detected in low concentrations in the systemic circulation of adult non-pregnant and pregnant sheep. Basal plasma concentrations of IR-GRP measured in the plasma of adult non-pregnant ovariectomized sheep were 27 ± 3 fmol/ml and this did not change significantly when the same animals were treated with E_2 given separately (26 ± 2 fmol/ml or together with P_4 (25 ± 4 fmol/ml; refer to Table 4). However, basal plasma

IR-GRP concentrations in pregnant sheep were 14 ± 2 fmol/ml; values significantly less than those measured in non-pregnant sheep either with or without steroid treatment ($p<0.05$).

9.3.3. Plasma IR-GRP Produced by Exogenous GRP Administration

Plasma IR-GRP concentrations were elevated significantly above baseline during the 30 min. infusion of GRP600 to adult non-pregnant and pregnant sheep (Fig. 39; $p<0.05$). Peak plasma IR-GRP elevations occurred at + 20 min. following the administration of GRP100 into both non-pregnant (44 ± 14 fmol/ml) and pregnant (9 ± 5 fmol/ml) sheep. Administration of the higher dose of GRP (GRP600) to non-pregnant ovariectomized sheep produced peak IR-GRP elevations of 499 ± 112 fmol/ml at + 20 min. and this was not altered significantly by steroid treatment (Fig. 39). In addition, steroid treatment did not alter the time course of the changes in plasma GRP concentrations that occurred during the infusion of GRP600 (Fig. 39). A similar dose of GRP (GRP600) administered to pregnant sheep produced significantly smaller elevations in circulating GRP concentrations compared to those produced in non-pregnant sheep (Fig. 39; $p<0.05$). Peak IR-GRP elevations that occurred in pregnant sheep as a result of IV administration of GRP600 were 149 ± 47 fmol/ml and were not significantly different than those which occurred when Glu & GRP600 was administered (Fig. 40; $p<0.05$). Administration of glucose alone (Glu) to pregnant sheep did not significantly affect circulating IR-GRP concentrations (Fig. 40).

In the fetus, plasma IR-GRP concentrations were not detectably altered from the very high basal concentrations in response to GRP600 adminstration (Fig. 39). In addition, plasma IR-GRP concentrations in fetal sheep were not altered by the administration of GRP100 or GRP600 to their pregnant mothers (data not shown). Similarly, there was no detectable change in maternal plasma IR-GRP concentrations following GRP administration to fetuses (data not shown).

9.3.4. Endogenous IR-GRP Concentrations In Relation to Feeding and Fasting

The concentration of IR-GRP measured in the plasma of non-pregnant ovariectomized sheep during and after feeding ranged between 20 -125 fmol/ml (Fig. 41). Plasma IR-GRP concentrations tended to increase over baseline within 2 hr of feeding and were lower when the animals were fasted. However, the changes detected in plasma IR-GRP concentrations following feeding were small and often below the limit of sensitivity of the RIA for plasma IR-GRP (8 fmol/tube) and the time and magnitude of the change in plasma IR-GRP concentrations following feeding varied greatly between and within sheep. As a result, no statistically significant change in plasma IR-GRP concentrations following feeding could be demonstrated. Plasma insulin concentrations (Fig. 41) as well as plasma glucagon and glucose concentrations (Fig. 42) tended to increase following feeding and were lower when the animals were fasted. However, these changes were not statistically significant.

9.4. DISCUSSION

In the present study I have demonstrated the presence of very high IR-GRP concentrations in plasma samples taken from d118-135 fetal sheep. As a result of these high fetal basal GRP concentrations, exogenous administration of relatively low doses of GRP did not alter significantly circulating GRP concentrations. IR-GRP was also detected within the systemic circulation of adult non-pregnant and pregnant sheep, however, IR-GRP levels were significantly lower in pregnant compared to non-pregnant sheep. IR-GRP concentrations rose following feeding of non-pregnant sheep but these rises seen following feeding failed to attain statistical significance. IV administration of GRP100 but not GRP600 to non-pregnant and pregnant animals produces circulating IR-GRP concentrations which are within the range of endogenous IR-GRP levels. However, peak

IR-GRP concentrations achieved following the infusion of similar doses of GRP were significantly lower in pregnant compared to non-pregnant sheep with or without steroid treatment.

In the present study, I was unable to demonstrate the presence of GRP in the pancreas of fetal and adult sheep using either immunocytochemical or immunoassay techniques, However, I have demonstrated using immunocytochemical techniques, IR-GRP-containing nerves in the GI tract of fetal and neonatal sheep.

The validity of the GRP measurements made in this study is dependent on the specificity of the antiserum used. In this study two different antisera were employed and both have been characterized extensively in previous studies (AB103; Ghatei et al., 1982 and LR-16; McDonald et al., 1988b). The AB103 antiserum was used to detect IR-GRP in the plasma of non-pregnant and pregnant sheep. This antiserum was raised in rabbits against bombesin and recognizes the C-terminal region of this molecule (Ghatei et al., 1982). McDonald and co-workers (1988b) have demonstrated the LR-16 and AB103 antisera both detect GRP and bombesin equipotently. However, studies to date have localized bombesin only in amphibians and GRP is recognized as its mammalian analogue (McDonald et al., 198[illegible] and 1983). Therefore it is unlikely that these antisera are detecting bombesin in the plasma of adult sheep. This antibody does however cross react 1-2% with other structurally similar peptides such as substance P and neuromedin B which are present in mammals (McDonald, personal communication). It is possible therefore that some of the IR-GRP detected in the sheep plasma represents the presence of either of these peptides. The concentrations of IR-GRP present in the circulation of adult sheep were very low but within the sensitivity of the assay used in this study.

The LR-16 antiserum was used in the determination of fetal plasma IR-GRP concentrations, as it is a particularly specific antiserum having less than 0.01% cross reactivity with structurally related substance P and other tachykinins and with neuromedin B. This antiserum has no detectable cross reaction with other structurally unrelated GI peptides (McDonald et al., 1988b).

The LR-16 antiserum used in immunohistochemical studies produced positive staining only in neural structures and not in other cell types which is consistent with GRP localization in other mammalian species (Ghatei et al., 1983; Brown et al., 1978; Walsh et al., 1979; and McDonald et al., 1981). Furthermore, IR-GRP staining was abolished following preabsorption of the antiserum with GRP. These results also strongly support the specificity of the staining of GRP-containing structures by the LR-16 antiserum.

Serial dilutions of fetal plasma samples produced a dilution curve which was parallel to synthetic porcine GRP standard suggesting that this RIA technique can accurately measure IR-GRP within the plasma of fetal sheep. Although the cross reactivity of many different known peptides have been examined and shown to be negliable (McDonald et al., 1988b), the possibility that the LR-16 is cross reacting with some as yet unidentified peptide cannot be ruled out at the present time. Further studies employing high performance liquid chromatography (HPLC) techniques are required before the identity of this IR-GRP species present in the plasma of fetal and adult sheep can be confirmed. In addition, it is not known whether high circulating concentrations of GRP present in the fetal plasma are bioactive or bound to circulating plasma binding protein.

Assuming that the IR-GRP detected in this study is in fact GRP, these results add significantly to other results in this thesis and stimulate interest in further investigation which will be discussed in the next chapter.

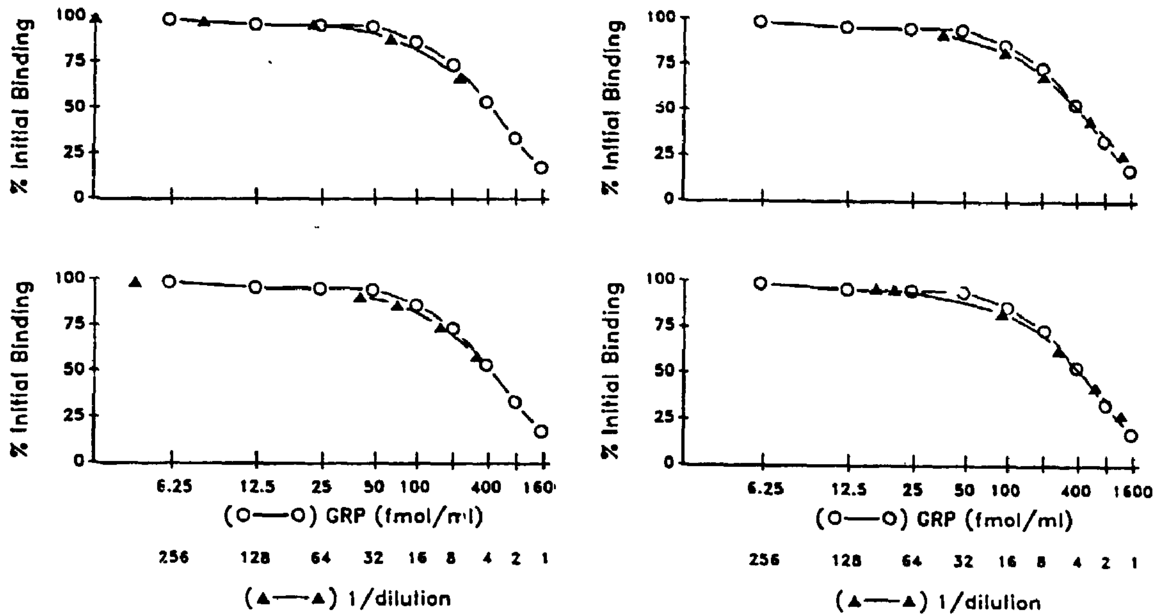

Figure 37. Mean of the percentage bound counts in serial dilutions of (O) synthetic porcine GRP standard and of (▲) four samples of fetal sheep plasma.

Figure 38. GRP immunoreactive nerve fibers (arrow) demonstrated in the myenteric plexus of the duodenum of a term fetus using LR-16 primary antiserum and localized with rhodamine fluorescent label (red) (X400).

	NON-PREGNANT			PREGNANT	FETAL
	NTX	P4E2	E2		
GRP (fmol/ml)	27 ± 3	26 ± 2	25 ± 4	14 ± 1	1708 ± 390

Table 4. Mean ± SEM of plasma immunoreactive GRP concentrations measured in preinjection samples collected from four d117-141 pregnant sheep and their fetuses as well as from four non pregnant ovariectomized sheep (NTX) not treated with steroids and the same animals treated with daily subcutaneous injections of (P4E2) both 50 mg of progesterone and 25 µg of estradiol (E2) or estradiol alone.

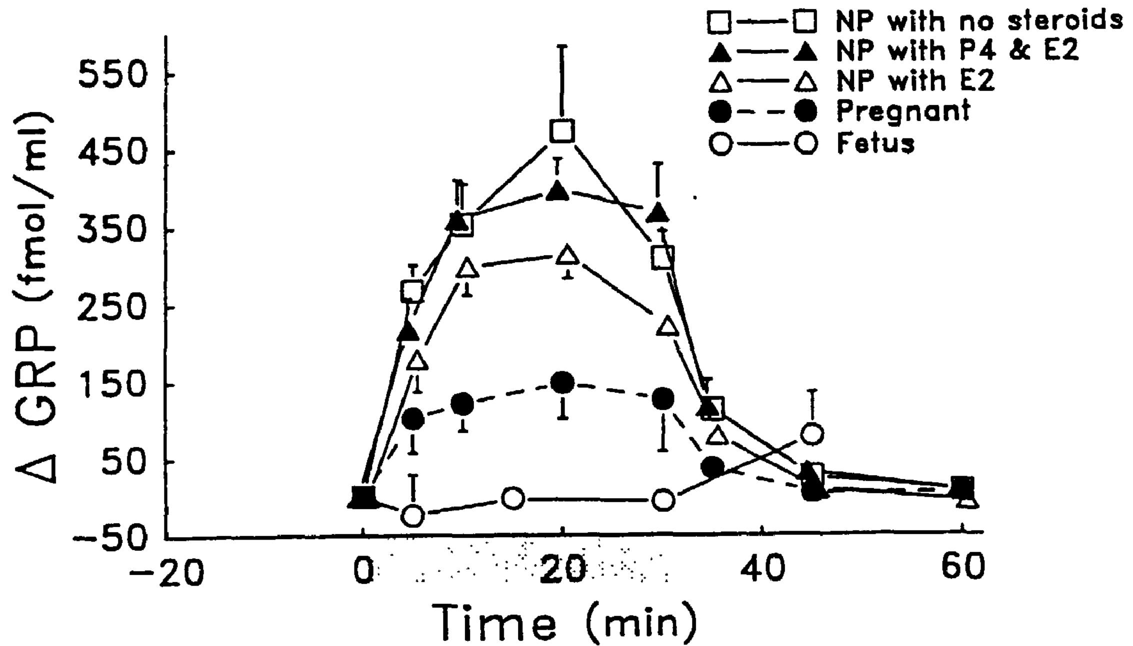

Figure 39. Mean ± SEM of the change over baseline of plasma IR-GRP concentrations following a 30 minute infusion of 600 pmol/kg·hr of GRP to (○ ; n=3) d118-135 fetal sheep, (● ;n=4) pregnant sheep, and four non-pregnant sheep (□) not treated with steroids, and given daily subcutaneous injections of (▲) both progesterone (50 mg) and estradiol (25 μg), and (△) estradiol (25 μg) alone.

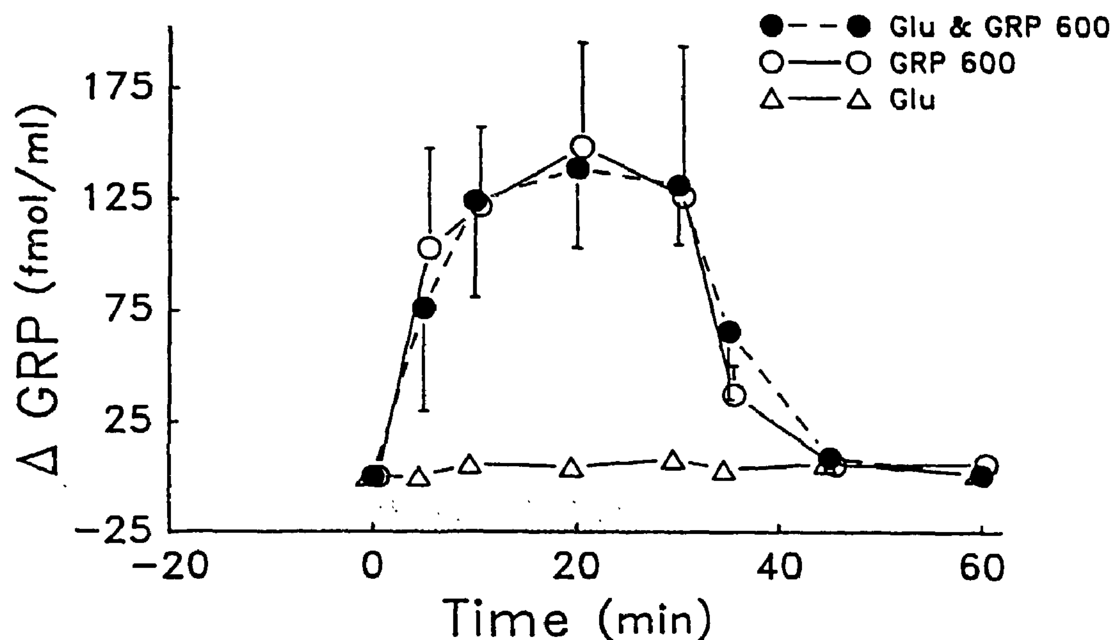

Figure 40. Mean ± SEM of the change over baseline of plasma IR-GRP concentrations measured in four d118-135 pregnant ewes following a 30 minute infusion of (Δ ; Glu) 10 mg/kg.min of glucose given alone, (O ; GRP600) 600 pmol/kg hr of GRP given alone, and (● ; GRP600 & Glu) 600 pmol/kg hr of GRP plus 10 mg/kg.min of glucose given together. (Where no SEM is shown, error is less than the size of the data point).

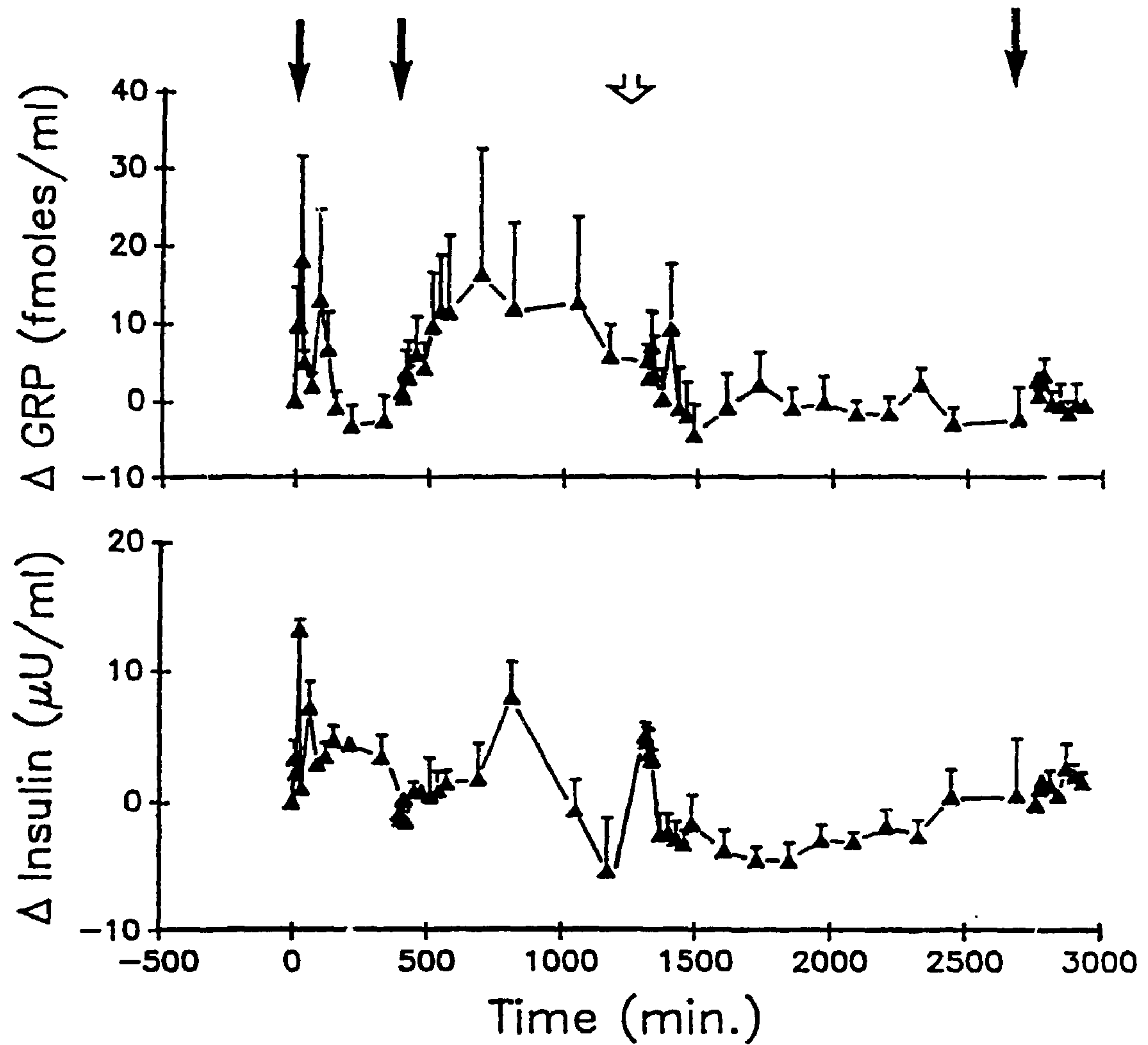

Figure 41. Mean ± SEM of the change over baseline of (top panel) plasma IR-GRP concentrations and (bottom panel) plasma insulin concentrations measured in non pregnant ovariectomized sheep during and after (↓) feeding and (⇩) sham feeding.

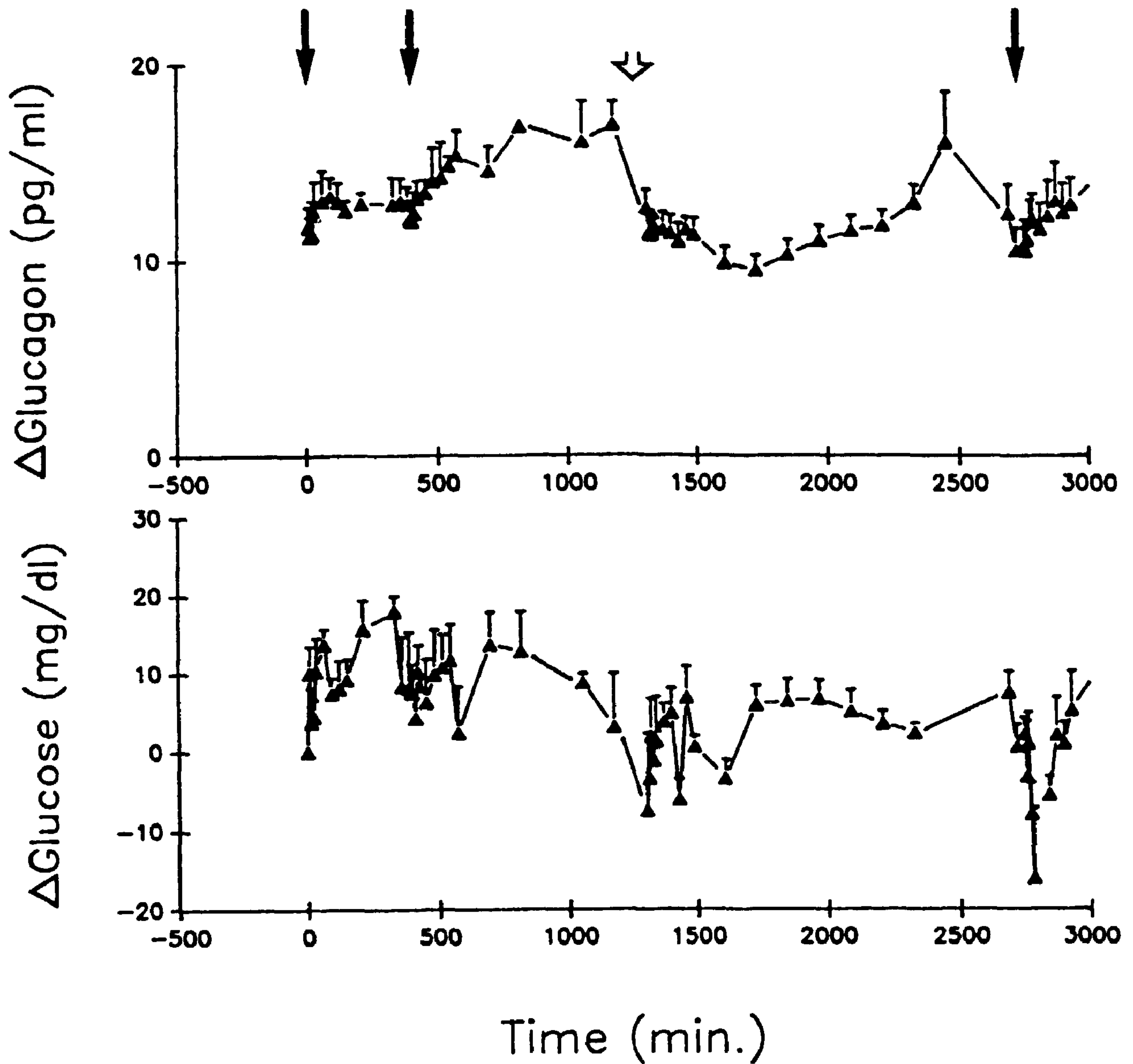

Figure 42. Mean ± SEM of the change over baseline of (top panel) plasma glucagon concentrations and (bottom panel) plasma glucose concentrations measured in non pregnant ovariectomized sheep before, during, and after (↓) feeding and (⇩) sham feeding.

10. GENERAL DISCUSSION

The experiments described herein have provided the following results:

1. Intravenous administration of GRP stimulates insulin and glucagon release in adult non-pregnant sheep.

2. Overnight fasting, which significantly reduced circulating glucose concentrations, produced a significant attenuation of GRP-stimulated insulin release in non-pregnant and pregnant sheep.

3. GRP potentiates glucose-stimulated insulin release in adult non-pregnant sheep.

4. The ability of GRP to stimulate insulin release and potentiate glucose-stimulated insulin release, is attenuated in pregnant compared to non-pregnant sheep.

5. The diminished insulin response to GRP given separately and together with glucose observed in pregnant sheep was reproduced in non-pregnant ovariectomized sheep treated with P_4 and E_2 but not with E_2 alone

6. An insulin response to an acute glucose challenge is present in fetal sheep over the last one-third of gestation.

7. GRP administration, at a dose which stimulated insulin release in the adult, did not produce significant elevations of plasma insulin levels in fetal sheep.

8. Elevation of fetal plasma glucose concentrations via concurrent glucose administration did not alter the lack of insulin response to GRP in fetal sheep.

9. IR-GRP was not detectable in the pancreas of fetal and adult sheep but was demonstrable in nerves located in the GI tract of fetal and neonatal sheep.

10. IR-GRP was detected in the systemic circulation of non-pregnant and pregnant sheep and preliminary studies suggest that IR-GRP concentrations rise in non-pregnant sheep following feeding.

11. IR-GRP is present in very high concentrations in fetal plasma.

The absence of IR-GRP containing nerves in the pancreas of adult sheep is consistent with results from previous immunocytochemical studies in other ruminant species (Bloom et al., 1983b; Dockray et al., 1979) but is in contrast to similar studies performed in several different monogastric species which have localized IR-GRP within the pancreas (Greeley et al., 1984; Ghatei et al., 1984) and certain studies have specifically demonstrated IR-GRP nerves intimately associated with the pancreatic islets in dogs (Buchan and McDonald, unpublished results).

IR-GRP immunoreactivity was however detectable within nerves of the GI tract of fetal and neonatal sheep and in the systemic circulation of adult non-pregnant and pregnant sheep. Therefore it is possible that in sheep, GRP is released from neural structures of the GI tract, or elsewhere, into the circulation through which it may stimulate insulin release by either acting directly on the islet cells or by indirectly stimulating the release of other insulinotrophic GI peptides. In addition, GRP may also act to alter glucose uptake and/or utilization in peripheral tissues. The proposed incretin-like action of GRP in sheep mediated by release into the systemic circulation is consistent with previous reports in other ruminant species (Bloom et al., 1983a,b) and would greatly contrast the local mechanism of GRP action which has been proposed to occur in monogastric animals. The reason for this significant difference in the mechanism of action of GRP between monogastric and ruminant animals is unknown. It is interesting to speculate that in ruminants GRP may supplant the role of GIP which is considered to be the most likely incretin candidate in monogastric species (Brown and Otte, 1978) but appears not to play a role in the control of insulin release in ruminants (Nilssen et al., 1983; Bunnet and Harrison, 1988).

To demonstrate that GRP is indeed acting as an incretin in sheep, the following criteria must be fulfilled: (1) that GRP is involved in an endocrine transmission between the gut and pancreas, (2) that GRP must be capable of augmenting glucose-stimulated insulin release when exogenously infused in amounts not exceeding physiological concentrations, and (3) GRP should be released into the systemic circulation by oral but not IV administration of glucose or nutrients (Creutzfeldt, 1976). The studies in this thesis have demonstrated that IR-GRP is present in the GI tract and systemic circulation but not in the pancreas; therefore it is possible that GRP is involved in endocrine transmission between the gut and pancreas. Furthermore, I have also demonstrated that GRP potentiates glucose-stimulated insulin release in adult non-pregnant sheep. Although IV administration of GRP600 produced supraphysiological concentrations of plasma IR-GRP, the lower dose of GRP (GRP100) produced circulating concentrations of IR-GRP that were within the range of circulating endogenous IR-GRP levels measured. Lastly, IR-GRP concentrations were not significantly altered during IV administration of glucose but plasma IR-GRP concentrations tended to rise following oral nutrient administration to non-pregnant sheep (feeding) and these elevations appeared to be associated with changes in plasma insulin concentrations. Rises in plasma IR-GRP seen following feeding of non-pregnant sheep are consistent with preliminary studies reported by Bloom and co-workers (1984) that report significant elevations in plasma IR-GRP concentrations following feeding of conscious calves. However, changes in plasma GRP concentrations following feeding detected in the present study in sheep did not attain statistical significance and therefore further studies are needed to confirm this last criteria for the incretin action of GRP in sheep. Thus most of the criteria have been fulfilled to suggest that in sheep, and in other ruminants, GRP is a strong incretin candidate involved in the endocrine transmission between the GI tract and pancreas. Whether or not the insulinotropic effect of GRP is

important in the physiological control of the endocrine pancreas of sheep awaits the acquisition of a specific and potent GRP antagonist.

It was previously suggested that decreased action of potentiators of glucose-stimulated insulin release, such as GRP, may be responsible, at least in part, for the development of glucose intolerance during pregnancy (H[illegible]nes et al., 1978). This suggestion has been supported by studies in this thesis which demonstrated that GRP administration alone or together with glucose resulted in significantly smaller elevations in plasma insulin concentrations in pregnant compared to non-pregnant sheep. However, administration of a similar dose of GRP per kg body weight to pregnant and non-pregnant sheep did not result in similar elevations in circulating IR-GRP concentrations. Lower circulating concentrations of IR-GRP produced in pregnant animals may be due, in part, to dilution of IR-GRP in the increased blood volume which occurs during pregnancy or it may be caused by increased rate of degradation of GRP in pregnant animals. The biological half-life of GRP in non-pregnant monogastric animals is 2-3 min. (Bloom et al., 1978). Studies examining the half-life of GRP in sheep have not been performed and whether or not this changes during pregnancy remains to be determined. Studies in this thesis demonstrated that basal IR-GRP concentrations are lower in pregnant compared to non-pregnant sheep which suggests that during pregnancy, either the GRP production rate is reduced or the metabolic clearance rate of GRP is increased. The attenuated insulin response to GRP observed in pregnant compared to non-pregnant animals is unlikely to be due simply to the lower circulating levels of GRP attained following exogenous GRP administration since treatment of non-pregnant ovariectomized sheep with physiological amounts of P_4 and E_2, but not E_2 alone, produced a similar diminished response. These results suggest that high circulating P_4 concentrations present during late gestation are, at least in part, responsible for the reduced insulinotrophic effect of GRP.

Interestingly, plasma concentrations were approximately 100 fold higher in the fetal circulation than those measured in either non-pregnant or pregnant adult sheep. As a result fetal plasma IR-GRP concentrations were not significantly altered during IV administration of either dose of GRP. It is not surprising therefore that no significant change in plasma insulin concentrations could be detected in response to these doses of GRP administered. Therefore we are unable to conclude from these studies whether or not GRP stimulates insulin release in fetal sheep. It is also possible that the lack of insulin response of GRP in the fetus is due to a lack of GRP receptors or a post-receptor deficit in the fetus. However, further experiments in which greater amounts of GRP are administered, will have to be performed before these possibilities can be addressed.

The site of production of GRP in the fetus is of interest. It is unlikely that substantial amounts of IR-GRP originate from the few nerves which were present in the GI tract. Endocrine cells containing IR-GRP have been previously demonstrated in human fetal lung (Ghatei et al., 1983) and therefore it is possible that this is the site of production in this species. Recently the production of numerous endocrine factors including GI peptides has been demonstrated in the placenta (Attai et al., 1984; Jones, 1989). Therefore it is also possible that GRP is produced in placental cells and secreted into fetal circulation. Further experiments must be conducted to answer these speculations.

Even more interesting is the question of the role of GRP in the fetus. Many GI peptides including GRP have been demonstrated to have tropic effects (Willey et al., 1984; Johnson and Guthrie, 1983; Lehy et al., 1983; and Sporn et al., 1985) and it therefore possible that GRP is an important factor in the control of proper growth and development of the GI tract, or perhaps other regions, during fetal life. Since results from this study demonstrate that GRP stimulates basal insulin release and potentiates glucose-stimulated insulin release in adult animals, it is possible that GRP stimulates insulin

release in the fetus. Furhter experiments must be performed to determine the role of GRP during fetal life.

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APPENDIX I - CONSTRUCTION OF CATHETERS

This appendix describes the procedure used in the preparation of catheters for the chronic fetal sheep experiments. The catheters were basically constructed from two calibers of vinyl tubing (V4, 1.2 mm o.d., 0.6 mm i.d. and V11, 2.3 mm o.d., 1.5 mm i.d, Bolab Inc., Lake Havasu City, Arizona).

Fetal vascular catheters consisted of a 3.8 cm aluminum blunt needle with a luer lock hub (20 gauge) which had been inserted into a length of V4 tubing (150 cm). The V4 tubing was then threaded through a piece of heavier V11 tubing (160 cm). The two layers of tubing were sealed at both ends with silicone adhesive (Silastic, Dow Corning Corp.) injected into the space between the catheters using a 5 ml syringe and an 18 gauge blunt needle. The heavier tubing provided additional support and reduced the likelihood of catheters becoming kinked in utero.

The amniotic fluid catheter consisted of 173 cm length of V11 tubing attached to a 15 gauge blunt needle with side holes cut into the last 2 cm. In order to reduce the chance of obstruction of the amniotic catheter tip, a "basket" was attached to the end of the V11 tubing. A 6 cm long segment of heavy weight vinyl tubing (slightly larger internal diameter than the external diameter of V11 tubing), with holes cut at irregular intervals around its circumference and length, was tied with silk suture to the end of the amniotic catheter. A longer segment of heavy weight tubing (8 cm) which had four longitudinal cuts extending to within 0.5 cm of the ends of the segment, was placed around the first piece and was compressed lengthwise to 6 cm (causing the four strips to bow outward) and then tied in place using suture.

The vascular catheters used in pregnant and non pregnant sheep were made

using a length of V11 (160 cm) tubing attached to a 15 gauge blunt needle and marked 10 and 20 cm from the tip. The marks assisted in estimating the length of catheter being inserted into the vessel during surgery.

The needle hubs were marked using a colour code so that catheters could be identified during and after surgery. The vascular catheters used in adult sheep were folded into envelopes which were then sterilized with gas using the hospital facilities. The fetal catheters and an amniotic fluid catheter were packaged to prevent contamination of the catheter tips (the region that will be located inside the amniotic cavity) and to allow them to be inserted through a hole in the flank of the ewe made using a trochar. The ends of the fetal catheters were aligned, and the last 15 to 20 cm of the catheter bundle was wrapped with "Saran Wrap" which was secured in place with silk suture. Fetal catheter sets were folded into envelopes and gas sterilized using hospital facilities.

APPENDIX II : COMPOSITION OF GRAIN PROVIDED TO THE SHEEP

MASTERFEEDS 14% EWE FITTING AND NURSING CHOW

Guaranteed Analysis:

Protein (min. %)....................................14.00

Crude fat (min. %)..................................2.50

Crude fiber (max. %)................................8.00

Salt (Act. %)...0.75

Sodium (Act. %)......................................0.30

Copper (Act. MG/KG)................................9.00

Vitamin A (min. IU/KG)............................10,000

Vitamin D (min. IU/KG)............................1,000

Vitamin E (min. IU/KG)...............................10

Calcium (Act. %).....................................0.80

Phosphorus (Act. %)................................0.50

Equivalent crude protein from non protein nitrogen 3.0%