1997

Regulation of carbohydrate metabolism by exogenous glucagon in lactating cows

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Regulation of carbohydrate metabolism by exogenous glucagon in lactating cows

by

Pengxiang She

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Nutritional Physiology
Major Professors: Jerry W. Young and Gary L. Lindberg

Iowa State University
Ames, Iowa
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GENERAL INTRODUCTION

Dissertation Organization

This dissertation is presented in the alternate format. It begins with a General Introduction that is followed by a Literature Review on topics related to the dissertation research. Then, two manuscripts that have been prepared from the data collected from the research project are included. These manuscripts were prepared for publication, and each one is complete in itself and has an abstract, introduction, materials and methods, results, discussion, conclusions, references, and acknowledgments. References are listed separately following each chapter of the dissertation. Finally, this dissertation is concluded with a General Summary, Appendix, and Acknowledgments.

Background and Objectives

The central aim of regulation of carbohydrate metabolism is to maintain glucose homeostasis, which results from the balance of glucose production and utilization. Both glucose production and glucose utilization are regulated elaborately by various mechanisms. For example, the liver, through regulation of hepatic glucose production and utilization (the latter mainly occurs in nonruminants), plays a pivotal role in maintaining glucose homeostasis. The liver is the major organ that performs gluconeogenesis. The flux of gluconeogenic substrates is controlled by both dietary and hormonal factors that mainly regulate key rate-controlling enzymes in the pathways of both gluconeogenesis and
glycolysis. Phosphoenolpyruvate carboxykinase (PEPCK) is one of those key regulatory enzymes. It has no known allosteric effectors and is regulated at the gene expression level, i.e., by transcription rates and stability of its mRNA. An abnormality in any regulatory mechanism may result in a disturbance of glucose homeostasis such as occurs in diabetes mellitus.

Because of the fermentative nature of the environment in the rumen, carbohydrate metabolism in ruminants differs from that of nonruminants in several aspects. Dietary carbohydrates are fermented to volatile fatty acids in the rumen rather than being digested and absorbed as glucose in the small intestine. As a result, the ruminants depend on continuous gluconeogenesis to provide glucose, which differs greatly from nonruminants. Also, because glucose is required for synthesis of milk lactose during the whole lactation period in lactating cows, the demands for glucose are great. Thus, gluconeogenesis in lactating cows is very important for maintaining both glucose homeostasis and milk production. Because glucose metabolism differs between ruminants and nonruminants, their regulatory mechanisms may also differ, but the mechanistic nature of the differences has not been clearly elucidated.

Lactation ketosis is a common metabolic disorder in dairy cows, and it occurs in the first several weeks after calving. Ketosis is associated with disturbances of both carbohydrate and lipid metabolism, and it is characterized by hypoglycemia, depletion of liver glycogen, hyperketonemia, and fatty liver. Ketosis generally is thought to result from endogenous glucose deficiency or low carbohydrate status. Early lactation cows are in a state of negative-energy balance, which indirectly causes increased lipolysis from adipose tissue.
As a result, ketogenesis increases when the carbohydrate status is low. The specific biochemical aberrations causing the pathogenesis of ketosis and fatty liver, however, are still unidentified. For example, it is still unclear whether gluconeogenic capacity is impaired in cows with ketosis and fatty liver. Also, the regulation of feed intake in early lactation cows is poorly understood, and the importance of decreases in feed intake in development of ketosis is unclear.

Glucagon, a polypeptide hormone containing 29 amino acids, is secreted by the pancreatic α-cells. Glucagon acts to regulate metabolism through specific signal transduction pathways that alter intracellular levels of cAMP and Ca²⁺. Glucagon is considered to be a catabolic hormone that regulates carbohydrate, lipid, and protein metabolism, but some aspects of its regulation in vivo are still uncertain, i.e., the lipolytic and proteolytic effects. The liver is the major target organ of glucagon, and one vital physiological role of glucagon is the maintenance of glucose homeostasis by increasing hepatic glucose production. Our general hypothesis for this dissertation research is that exogenous glucagon infused into dairy cows would increased the availability of endogenous glucose without also increasing the net availability of endogenous NEFA mobilized from adipose tissue and that hormonal regulation of gene expression for gluconeogenic enzymes is critical in controlling glucose homeostasis in response to hormonal status, as well as in development of ketosis in dairy cows.

The present studies were undertaken mainly to gain further understanding of the regulation of carbohydrate metabolism by exogenous glucagon in lactating cows. Specific objectives were:
1. To determine pharmacological effects of long-term intravenous infusions of glucagon in normal, early-lactating dairy cows with an emphasis on regulation of glucose metabolism.

2. To examine changes of mRNA for selected gluconeogenic enzymes, along with other metabolic parameters, caused by glucagon infusion into normal lactating dairy cows for both long-term and short-term periods.

3. To measure PEPCK mRNA and its response to glucagon infusion in cows that had fatty liver and were susceptible to ketosis.
This review of literature consists of five parts: 1) etiology of lactational ketosis, 2) fatty liver and its relationship with ketosis, 3) gluconeogenesis in ruminants, 4) regulation of gene expression of PEPCK, and 5) glucagon in ruminants. These are broad areas of discussion, and my intention is to provide an overview about the nature of ketosis, fatty liver, gluconeogenesis, and glucagon in dairy cows. Also, the regulation of PEPCK is reviewed at the molecular level.

Etiology of Lactational Ketosis

**Definition of ketosis**

In dairy cows, ketosis is a major metabolic disorder, which generally is thought to be caused by disturbances of carbohydrate and lipid metabolism. Ketosis is characterized by hyperketonemia and hypoglycemia. Depending on the degree of clinical signs, there are two forms of ketosis, clinical and subclinical. Signs of ketosis include loss of appetite, decreased milk production, and rapid loss of body weight. Some cows become excitable; however, the majority is apathetic. The general biochemical changes of spontaneous ketosis are increases in plasma ketone bodies, NEFA, and acetate (8); decreases in plasma glucose, triglycerides, cholesterol, and phospholipid (129, 215); fatty infiltration of the liver (173); loss of liver glycogen (149); increased milk fat content, and decreased milk protein content (184).

Subclinical ketosis may be defined as a preclinical stage of ketosis, which is characterized by
elevated levels of ketone bodies without clinical symptoms. Total plasma ketone bodies in normal cows were less than 10mg/dl (174), but the lower and upper limits of ketone body concentrations used to specify subclinical ketosis are not consistent among investigators. Both plasma acetoacetate and BHBA have been used as indicators of subclinical ketosis. Some researchers have measured only milk acetone. Subclinical ketosis is can be costly because the incidence can be high and it may remain undetected; yet, it has adverse effects on productivity. Ketotic cows may recover spontaneously without treatment when milk production decreases rapidly.

**Incidence and risk factors**

Because it is not easy to differentiate between subclinically and clinically ketotic cows in field studies, most investigators have reported the prevalence of hyperketonemia, which includes both subclinical and clinical cases. The occurrence of at least one episode of hyperketonemia in cows during the first two months of lactation ranged from 7 to 32 percent (2, 54, 55 72, 77, 82, 84, 105). The incidence is significantly different between herds (3, 54, 87), which may be related to management, nutrition, or other factors such as week of lactation, breed, parity, season, and milk production. Ten years ago, the peak prevalence usually appeared during the fourth week of lactation; however, recent studies and experiences of veterinarians indicate that the peak time seems to have moved to the first week of lactation. For instance, Duffield et al. (55) reported a survey of 1,373 dairy cows in the Ontario area. They defined subclinical ketosis as a serum BHBA concentration of 1.2 mM or higher, and the prevalence of hyperketonemia in early lactation cows was about 14%, which
had changed very little over the previous 10 years (54). The prevalence peak, however, has moved from the fourth week of lactation to the first two weeks.

Ketosis usually occurs in high-producing cows. Concentrations of ketone bodies are positively associated with milk yield around the peak of lactation (3, 89, 105) and with milk yield during the entire lactation (57). Obesity or overfeeding cows with grain during the dry period increased the risk to have ketosis and other peripartal diseases (68, 128, 165, 188). In Scandinavian countries, high butyric acid content in silage was found to be an important factor leading to occurrence of ketosis (3, 199). Butyrate is absorbed through the rumen wall and converted to BHBA in the rumen epithelium.

Epidemiological studies have shown a close relationship between ketosis and other periparturient diseases. Among 61,124 Finnish Ayrshire cows, Grohn et al. (80) reported 15 different disorders that increased the odds for diagnosis of ketosis. The disorders included parturient paresis, abomasal displacement, hypomagnesemia, retained placenta, metritis, mastitis, foot or leg problems, rumen acidosis, and traumatic reticuloperitonitis. Markufeld et al. (126), reported no association between ketonuria and either milk fever or mastitis, however, significant associations were established between ketonuria and retained placenta, primary metritis, displaced abomasum, and aciduria after calving. It is thought that the periparturient diseases associated with ketosis cause loss of appetite of cows and induce ketosis; therefore, this type of ketosis is classified as secondary ketosis. The relationship between ketosis and abomasal displacement may be bidirectional rather than unidirectional (80).
Pathogenesis

While the physical characteristics of lactation ketosis in cows have been well established, the etiology of ketosis is still not clearly elucidated. It was well known that under fed conditions, correlations between NEFA and glucose or ketones are low (160). However, under fasting conditions or in cows in the early stages of ketosis, there is a significant negative correlation on a within-cow basis between glucose and NEFA (-0.66) and glucose and ketone bodies (-0.68), with a significant positive correlation between NEFA and ketone bodies (0.85) (159). It is clear that NEFA are an important source of blood ketones, with conversion occurring in the liver under either fasting or ketotic conditions. The negative correlation between blood glucose and both NEFA and ketones suggests that low availability of glucose is an important factor in the development of ketosis.

During early lactation, the metabolic priority given to the demands of milk production at a time when feed intake is limited was proposed to be a key factor in rendering dairy cows susceptible to spontaneous ketosis (9). Feed intake during this period is frequently insufficient to meet the combined metabolic demands of maintenance and milk production, so that cows are in negative energy balance. The metabolic priority for demands of milk production is probably established by changes in patterns of hormone secretion during the periparturient period. Lipolytic hormones promote lipid mobilization from adipose tissue for energy supply; thus, concentrations of plasma NEFA increase. Usually, NEFA began to increase two weeks before calving and peak by calving (81). However, hyperketonemia and hypoglycemia usually occurred in one to three weeks after calving (185, 189). The time course of changes in these metabolites may provide insights about the occurrence of ketosis.
Elevated plasma NEFA provide substrates for ketogenesis, but probably severe carbohydrate insufficiency is the precipitating factor for clinical ketosis (9).

A glucose deficiency could result from an imbalance between glucose demand for lactation and hepatic glucose production from gluconeogenesis. Deficiencies in glucose supply could be caused by inadequacies in availability of gluconeogenic precursors or by impairment of the gluconeogenic process. Although in vitro results from experimental ketosis showed impaired gluconeogenesis in the liver of ketotic cows (134), there is no in vivo study that directly measure gluconeogenesis of ketotic cows. Propionate loading tests show decreased conversion of propionate to glucose in spontaneously ketotic dairy cows (79); however, the propionate loading test is not a measure of in vivo gluconeogenesis in the liver. In addition, Smith (193) found that propionate loading was not affected by liver fat concentration. Studies on the activities of some key gluconeogenic enzymes are not conclusive, and the results are inconsistent, which will be discussed in detail in another section. The relatively low supply of gluconeogenic precursors compared with high demands for glucose for milk production might be a very important factor in initiating ketosis in early lactation cows, but very few studies have been conducted to approach this in depth.

In 1966, Krebs (108) proposed that oxaloacetate (OAA) deficiency is the underlying cause of ketosis. When the demand for glucose is great, OAA in the tricarboxylic acid (TCA) cycle is removed from the mitochondria for gluconeogenesis. If OAA concentration in the mitochondria becomes sufficiently depleted, there will not be enough OAA to condense with acetyl-CoA to allow initiation of the TCA cycle. When this happens, acetyl-CoA accumulates in the mitochondria and is removed via production of ketone bodies. Thus,
gluconeogenesis, by virtue of its utilization of OAA, promotes ketoneogenesis.

Concentrations of OAA and many other gluconeogenic intermediates decline in ketotic livers (13), but the intramitochondrial concentration of OAA has not yet been determined.

Components of the TCA cycle may be depleted by excessive gluconeogenesis, by a limited supply of precursors, or by a more reduced redox potential. A study by Wieland et al. (209) showed that an increased mitochondrial redox potential influenced the OAA:malate couple in favor of malate. In addition, Ballard et al. (16) showed that NADP-malate dehydrogenase activity was decreased in mitochondria, but activity of the enzyme within cytosol was increased during spontaneous ketosis. Thus, malate leaving mitochondria would supply precursors for gluconeogenesis, which deplete TCA cycle intermediates in mitochondria.

Ketogenesis in ruminants could be regulated in some key steps as in nonruminants. Ketogenesis may be regulated by transport of NEFA from plasma to the liver. Uptake of NEFA by the liver is regulated by both their concentrations and by rates of blood flow (61). The entry rate is determined also by concentrations of malonyl-CoA, which inhibits carnitine palmitoyl transferase I. This mechanism is very important for control of ketogenesis in nonruminants (130). Malonyl-CoA also inhibits carnitine acyltransferase in both bovine and sheep liver (30). Zammit et al. (218) pointed out that the decreased hepatic concentration of malonyl-CoA, which is probably caused by hypoinsulinemia and hyperglucagonemia in late gestation and early lactation, stimulated oxidation of fatty acids to ketone bodies. The role of malonyl-CoA in the development of ketosis needs further research because lipogenesis in the ruminant liver is low or absent. Also, recent evidence in nonruminants suggests that β-hydroxy-β-methylglutaryl-CoA synthase is an important control point for ketogenesis, and
possibly for β-oxidation, because of its highly regulated nature (136). In ruminants, propionate is an anti-ketogenic. Propionate inhibits oxidation of fatty acids by forming a nonfunctional adduct between flavin adenine dinucleotide and fatty acyl-CoA dehydrogenase (187). Succinyl-CoA generated from propionate in the mitochondria also inhibits ketogenesis by succinyllating β-hydroxy-β-methylglutaryl-CoA synthase (119).

Fatty Liver and Its Relationship with Ketosis

**Characteristics of fatty liver**

Fatty liver in dairy cows may be defined as a periparturient metabolic disorder characterized by fatty infiltration of the liver. Jasper (99) observed the livers of 2,400 cows at slaughter and found that 25% of recently calved dairy cows and 40% of dairy cows in late gestation had livers with visible evidence of fat accumulation, whereas less than 1% of cows in other stages of lactation had such evidence. Reid (162) reported that 100% of clinically normal cows demonstrated histologically discernible fat in the liver in the first week postpartum, and 63% of these cows had moderate to severe fatty liver. In addition, Reid et al. (163) estimated that one-third of high yielding dairy cow in the United Kingdom are affected by subclinical fatty liver. Based on histological data, he suggested that cows with less than 20% fat in the liver at one week after calving are considered normal and cows with more than 20% fat are considered to have fatty liver. Those percentages of fat were measured by using histologically stained sections, and the values are approximately twice as high as those determined by chemical assay. From the reported studies, it became obvious
that fatty liver occurs at a high incidence in periparturient cows. The adverse effects of fatty liver probably depend on the intensity of fat infiltration in the liver.

Cows with extreme fatty liver usually show a poor health status in the immediate postpartal period. The cows usually have obese body conditions, and clinical signs of fatty liver are complicated with the common postpartal diseases: metritis, mastitis, hypocalcemia, displaced abomasum, retained placenta, and ketosis. This situation is known as “fat cow syndrome” (138). Cows with fatty liver are leukopenic, with decreases in neutrophils, eosinophils, and lymphocytes (164) and show decreased fertility (118, 166). Treatments are usually ineffective, and prognosis is poor.

The most common cases of fatty liver are subclinical. The cows have abnormal fatty infiltration without obvious clinical symptoms. The exact chronological pattern of liver fat accumulation in dairy cows is not clear with peak triacylglycerols (TAG) content in the liver occurring at near caving (87), one week (162), or two weeks (203) after calving. Gerloff et al. (71) reported that concentrations of liver fat increased during the late dry period and peaked within the first three weeks of lactation. The inconsistency may be caused by different frequencies of biopsy, or it may be related to differences in herd management or severity of fatty liver among herds.

Fatty livers have rounded edges, and they are enlarged and discolored. Discoloration varies from a pale appearance in cases of mild fatty liver to a yellow-orange color in cases of severe fatty liver. In severe fatty liver, accumulation of TAG in parenchymal cytoplasm is accompanied by disruptions in hepatic structure, including fatty cysts in liver parenchyma, increased cell volume, compression of sinusoids, deceased volume of rough endoplasmic
reticulum, and mitochondrial damage. These morphological changes are all reversible, and there is no evidence of long-term damage to hepatocytes that results from fatty infiltration (138,163).

Liver TAG concentrations are positively correlated with concentrations of serum NEFA (167), but not with serum and urine ketone body concentrations or with blood glucose concentrations (69). Serum activities of hepatocellular enzymes, including aspartate aminotransferase (AST), ornithine decarboxylase (OCT), γ-glutamyl transferase, sorbitol dehydrogenase, and iditol dehydrogenase, are usually increased in cows with fatty liver, and AST is the enzyme that has been best correlated with fatty liver (106, 88, 193, 208). Neither these enzymatic activities nor bromsulphthalein clearance test are reliable indicators for diagnosis of fatty liver (87). The most reliable method of diagnosis of fatty liver is liver biopsy followed by chemical analysis.

Pathophysiology

Three major predisposing factors for development of fatty liver are 1) overcondition or obesity during the dry period, 2) rapid weight loss around calving and in early lactation, and 3) high milk yield (71, 138, 163). Dry matter intake of obese dry cows is more likely to be depressed during the periparturient period (68, 165). Most high-producing cows are in negative energy balance in early lactation. All these factors would cause rapid body loss and increased plasma NEFA, which are precursors for hepatic TAG synthesis and closely associated with development of fatty liver in dairy cows. In addition, low serum insulin concentrations and increased lipolytic hormones such as growth hormone, placental lactogen.
and prolactin during the periparturient period could promote mobilization of adipose TAG to provide additional energy and thereby increase plasma NEFA concentrations.

De novo fatty acid synthesis does not occur in ruminant liver (17). Any NEFA mobilized from adipose tissue are taken up by the liver from the plasma and can be either esterified to TAG, completely oxidized to CO₂, or partly oxidized to ketone bodies. Oxidation and ketogenesis from NEFA in the liver have been discussed already. In nonruminants, rates of fatty acid esterification and secretion of TAG as very low density lipoproteins (VLDL) increase simultaneously during periods of high fatty acid uptake (90), which does not occur in ruminants. Secretion of TAG by hepatocytes from pregnant or lactating sheep is about 60% greater than by hepatocytes from nonpregnant sheep, but this was still less than 2% of fatty acid uptake, whereas oxidation accounted for about 12% of uptake (62). Rates of TAG formation in ruminant liver are similar to those in other species. TAG secretion, however, is very small but detectable in cows (157, 156). A direct comparison among species of TAG secretion rates from liver slices demonstrated a relationship between dependence on the liver as a site for lipogenesis and ability of the liver to secrete TAG (157). Grummer (81) proposed that ruminants may have evolved without the mechanisms necessary for high rates of VLDL secretion because of the limited precursors for TAG synthesis. Little research has been conducted to determine the rate-limiting step for TAG export in ruminants, and the reason for “an inherently slow rate of VLDL secretion” is not known.

In vitro studies have shown that rates of fatty acid esterification were lower in hepatocytes from energy-restricted lactating goats (5), in the liver from feed-deprived
lactating cows (55), or in the perfused liver from feed-deprived pregnant sheep (44).

Recently, Van den Top et al. (203) found that activity of the enzyme for TAG synthesis, glycerolphosphate acyltransferase (GPAT), is significantly lower in fatty liver than in normal liver. This result confirmed earlier in vitro studies (5, 44, 55) and suggests that low GPAT activity may divert fatty acids from esterification to β-oxidation or ketone bodies to protect hepatocytes against further accumulation of TAG.

**Ketosis and fatty liver**

Ketosis and fatty liver have long been considered to be related metabolic disorders. The relationship between fatty liver and ketosis and the contribution of impaired carbohydrate and lipid metabolism toward the etiologies of these disorders, however, are not easy to elucidate because under field conditions it is hard to predict accurately which cows will succumb to these disorders. Spontaneous ketosis is usually accompanied by with fatty liver. Two lines of evidence indicate that fatty liver precedes ketosis. First, As already discussed, fatty liver is prevalent from calving to the first week postpartum, whereas cows are most susceptible to ketosis at three weeks postpartum. Second, A series of experiments from our laboratory also support the idea that fatty liver precedes ketosis, or more specifically, that ratios of TAG to glycogen in the liver greater than 2.5 seem to be required for occurrence of clinical ketosis (55).

In summary, although the specific biochemical aberrations are unidentified, fatty liver results from excessive esterification of NEFA and a reduced TAG secretion rate from the liver. Ketosis probably results from increased ketogenesis because of decreased carbohydrate
status in the liver. The decreased carbohydrate status could result from either low substrate availability or low gluconeogenic capacity. As already discussed, it is still unclear whether fatty liver impairs the gluconeogenic capacity of the liver. The glucose deficiency theory of ketosis is based on no defects of gluconeogenic enzymes and excessive gluconeogenesis in the liver (108). Gluconeogenic and ketogenic rates are both high in liver slices from early lactation cows (1). A liver with a high content of fat may divert fatty acids from esterification to β-oxidation (203) and may increase ketogenesis if the carbohydrate status in the liver is low at the same time. This hypothesis agrees with the chronology of development of fatty and ketosis and with physiological and pathological states of cows in the periparturient period.

Gluconeogenesis in Ruminants

*Introduction to regulation of glucose homeostasis*

It is important to maintain relatively constant blood glucose concentrations. Therefore, the body must have precise mechanisms to regulate glucose production via gluconeogenesis and glycogenolysis, cellular uptake of plasma glucose, and utilization of glucose for glycogen synthesis and for glycolysis. The nutritional and hormonal regulation of processes involved in glucose metabolism has been an active area of research for decades, and mechanisms for homeostatic regulation of glucose in nonruminants have been clarified gradually (73, 91, 151, 152). Gluconeogenesis and glycolysis have many common reactions in their pathways except for three futile cycles: the glucose/glucose-6-phosphate cycle, the
fructose-6-phosphate/fructose-1,6-bisphosphate cycle, and the pyruvate/phosphoenolpyruvate cycle. Hepatic glucose production and utilization involve movement of substrates through these three cycles, so they are thought to be the major regulatory sites for glucose metabolism (Figure 1). Pilkis and Claus (152) divided hormonal regulation of glucose metabolism into short-term, which involve alterations in enzyme activity brought about by changes in allosteric effectors and phosphorylation/dephosphorylation of enzymes that can occur in seconds to minutes, and the long-term effects that involve changes in gene expression, a process that usually takes hours to days.

In nonruminants, how gluconeogenesis is regulated during physiological states such as fasting, long-term starvation, diabetes, refeeding, and insulin or glucagon injections is well known. For instance, for acute regulation of enzymatic activities during fasting, glucagon or a β-adrenergic agonist binds to its plasma membrane receptor and activates adenylyl cyclase, which results in increased intracellular cyclic adenosine monophosphate (cAMP) concentrations. Cyclic AMP activates protein kinase A (PKA), and PKA phosphorylates 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2), a bifunctional enzyme. Therefore, the intracellular level of fructose 2,6-bisphosphate (Fru-2, 6-P₂), which is a strong inhibitor of fructose 1, 6-bisphosphatase (FBPase), is decreased. Fru-2, 6-P₂ is decreased due to inhibition of 6-phosphofructo-1-kinase (PFK) and activation of FBPase. The decrease in Fru-2, 6-P₂ inactivates pyruvate kinase (PK), both allosterically and by making the enzyme a better substrate for phosphorylation by PKA. These actions decrease PK activity and favor gluconeogenesis. On the other hand, after refeeding, insulin
Figure 1. Simplified pathways for gluconeogenesis (solid line) and glycolysis (dotted line) with an emphasis on regulatory sites in the liver. Gluconeogenic and glycolytic enzymes are indicated in boxes, and abbreviations are identified in text.
counteracts the stimulation of gluconeogenesis by glucagon and β-adrenergic agonists, probably by suppressing the rise in cAMP brought about by these hormones. Thus, insulin decreases the extent of phosphorylation of PK and PFK-2/FBPase-2 and decreases rates of gluconeogenesis.

Hormones also exert important long-term effects on glucose metabolism by changing rates of enzyme synthesis. These effects are mediated through alterations of rates of mRNA synthesis and, in some cases, by changes in the rate of degradation of specific mRNA. Gene expression may be mediated at the mRNA translation level; however, no evidence exists for this in enzymes of glucose metabolism. Complete cDNA molecules have been cloned for all of the cytosolic enzymes involved in the three substrate cycles of hepatic glucose metabolism. These enzymes include glucokinase (GK), glucose 6-phosphatase (G6Pase), PFK, FBPase, PFK-2/FBPase-2, PEPCK, PK, and pyruvate carboxylase (PC). Extensive research has been done on nutritional and hormonal regulation of gene expression of some of these enzymes in nonruminants. In ruminants, however, studies on enzymatic regulation of gluconeogenesis are limited. One reason for this limitation is that no bovine hepatocyte cell line has been available, and primary cell cultures of adult ruminant hepatocytes are difficult to maintain for unknown reasons.

Significance of and methods to study gluconeogenesis in ruminants

There are four major differences in glucose metabolism between ruminants and nonruminants. First, because most dietary carbohydrate is fermented in the rumen and only small amounts of glucose are absorbed directly from the gastrointestinal tract in ruminants.
90 to 100% of the glucose supply depends on a state of continuous gluconeogenesis. Second, in ruminants, gluconeogenesis increases after feeding when substrates are most plentiful, and it decreases during fasting. This is opposite to nonruminants where maximal gluconeogenesis occurs during fasting. Third, there are great demands in lactating cows for glucose, which is mainly used for synthesis of lactose in milk. Depending on the level of milk production, up to 80% of total glucose production could be used for milk production. Fourth, ruminants oxidized only limited amounts of glucose, and they use acetate instead of glucose for fatty acid synthesis. Acetate and butyrate are important sources of energy that spare glucose. Because activities of hepatic GK are very low, it has been proposed that glucose utilization in ruminant liver is limited (14). From the four differences, it is obvious that gluconeogenesis is very important in ruminants for maintaining both plasma glucose concentrations and milk production. Some unanswered questions regarding gluconeogenesis and its regulation in lactating dairy cows are as follows: 1) How much glucose is synthesized in the liver per day, and how much is derived from different gluconeogenic precursors? 2) How does gluconeogenesis change in different stages of lactation and in pregnancy? 3) How is gluconeogenesis regulated enzymatically? 4) How much is it affected by the availability of precursors and by hormones? To date, complete answers are not available for these questions, and some of the available research data are not consistent.

Methods for studying gluconeogenesis in ruminants include in vivo whole body kinetic studies and in vitro studies such as liver slice incubations, recently established primary hepatocyte cultures, and determination of enzyme activities. Two principal techniques have been used for quantitative estimates of rates of gluconeogenesis in vivo:
isotope dilution and arterio-venous catheterization. The use of isotope dilution or tracer techniques allows measurement of the rate of turnover or irreversible loss of metabolites in steady-state conditions. Double-isotope techniques are useful to simultaneously measure glucose turnover, substrate turnover, and the incorporation of substrate into glucose. Arteriovenous catheterization allows studies of individual organs in vivo. The in vivo techniques are not without disadvantages: for example, isotope dilution underestimates the gluconeogenic potential of a precursor because of label crossover in the TCA cycle. A relative new technique is the use of molecular biology tools to study metabolic regulation (74), but very little research has been done with this technique in ruminants.

**Gluconeogenic substrates**

Glucose is synthesized from propionate, glycerol, amino acids, and lactate. The contribution of each of these components to total gluconeogenesis is variable depending upon types of diet, amounts of diet, and physiological states of the animal.

*Propionate.* The major gluconeogenic precursor in fed ruminants is considered to be propionate absorbed from the rumen. About 90% of absorbed propionate is taken up by the liver (24). Studies have shown that 27 to 60% of the total glucose utilization is synthesized from propionate in sheep (103, 113, 195). Wiltrout and Satter (212) showed that 45% of total glucose was derived from propionate in lactating cows. Glucose infusion caused a decreased rate of glucose production from propionate, but the percentage of propionate converted to glucose did not change, and exogenous glucose was more effective at decreasing glucose production from other gluconeogenic precursors than from propionate (103). Little glucose
was derived from propionate in cows fasted for 4 days because it would only be absorbed in small quantities from the rumen (117).

**Glycerol.** Glycerol is found primarily as the backbone of TAG in adipose tissue, and concentrations of glycerol in plasma are low (23). Gluconeogenesis from glycerol is small in fed ruminants and contributes less than 5% of total glucose production (115). In fasted and ketotic sheep, however, up to 30% of irreversible loss of glucose was accounted for by glycerol (23). Ranaweera et al. (161) have shown that 13% of total glucose entry is derived from glycerol in normal pregnant sheep, 17% during starvation, and 91% during glycerol infusion. These data suggest that glycerol can be an important source of glucose production when its concentrations increase because of lipid mobilization during negative energy balance.

**Amino acids.** Contributions of amino acids to gluconeogenesis also are variable, depending upon physiological and nutritional status. Individual amino acids used for gluconeogenesis, listed in descending order of contribution, are alanine, glutamate, aspartate, serine, and glycine (213). Wolff et al. (213) used in vivo kinetic techniques to estimate that 11 to 30% of irreversible loss of glucose is derived from amino acids in fed sheep. Reilly et al. (168) reported a 28% contribution in cows, and Egan and Black (58) estimated that between 30 and 50% of glucose is derived from lactate, glycerol, and amino acids combined in maintenance-fed steers. Again, in vivo kinetic experiments often are difficult to interpret because of cross-over of labeled carbon atoms.

**Lactate.** Muscle and adipose tissue produce lactate endogenously, and it can be a significant product of soluble carbohydrate in the rumen. The liver and kidney normally are
net users of lactate. Lindsay (115) estimated that lactate contributed 28% of total glucose, which is after correction for crossover of carbon from lactate. Studies with fed sheep showed only a 4 to 10% contribution of lactate to glucose (24), but in starved sheep there was about a 15% contribution of lactate (4). The fraction of lactate turnover converted to glucose has been calculated to be 30 to 40% in pregnant sheep and lactating cows, but it was only 10% in lactating sheep and 27% in pregnant cows (12). In addition, the glucose-lactate interconversion, namely the Cori cycle, seems to be lower in ruminants than in other species (12).

**Nutritional and enzymatic regulation of gluconeogenesis**

Because glucose metabolism differs between ruminants and nonruminants, regulatory mechanisms may also differ, but the mechanistic nature of the differences has not been clearly elucidated. In general, gluconeogenesis is controlled by substrate availability and by rate-limiting enzyme activities.

The availability of substrates is a major factor determining the rate of gluconeogenesis (115). Positive correlations between digestible energy intake and glucose entry rates have been reported by Leng (112), Lindsay (114), Steel and Leng (195), Herbein et al. (86), Schmidt et al. (183), and Yost et al. (216). Rates of glucose synthesis in sheep were increased by ruminal infusions of propionate and casein, and it was suggested that the rate of gluconeogenesis be related directly to the availability of gluconeogenic precursors absorbed from the digestive tract (102). Veenhuizen et al. (205) fed propionate at 6.3 mol/d to steers and found an increase of 1.3 mol/d in glucose production. Gluconeogenesis
decreases when substrate availability becomes limiting. Lindsay (114) reported a 33% decline in glucose production after a 24-h fast, and Katz and Bergman (104) reported a 50% decline in glucose production in sheep fasted for three days.

The results of in vitro measurements of activities of gluconeogenic enzymes in ruminant livers from studies in the 1960s and 1970s varied from each other and also differed from activities in the liver of nonruminants. Activity of PC increased during fasting in sheep (64) and in lactating cows (16). Baird et al. (13) showed that PC activity increased in spontaneously ketotic cows, whereas Ballard et al. (16) did not detect the increase. In diabetic sheep, significant increases in PC and PEPCK activities were shown by Filsell et al. (64). Administration of dexamethasone caused a significant decrease in PC activity in sheep (64), but no changes were found in lactating cows (10). Glucagon infusion in sheep caused an increase in PC activity without changes of PEPCK, FBPase, and G6Pase (31). Compared with nonlactating cows, PC activity (16) and FBPase (132) activity in lactating cows are significantly greater. Similarly in sheep, Smith et al. (192) has shown that PC activity increased 5- to 7-fold and FBPase activity increased two fold during late pregnancy and in early lactation when compared with nonpregnant sheep. Smith et al. (1982) also found that PFK activity decreased by 50% during lactation, that PK activities declined by about 40% at the end of pregnancy, and that PK activity is very high in sheep. These results suggested decreased recycling of fructose-6-phosphate and phosphoenolpyruvate and enhanced gluconeogenic capacity in late pregnancy and early lactation in sheep.

Only one study has shown a significant decrease in PEPCK activity in fasting lactating cows (14). During starvation, no significant changes were found in PEPCK
activities in sheep (64), lactating cows (16), or Holstein heifers (217). Also, no significant changes have been found in spontaneously ketotic cows (13, 16). Dexamethasone caused a significant decrease in PEPCK activity in spontaneously ketotic cows (11) and in normal lactating cows (10). All-concentrate diets caused a 24% increase in PEPCK activity in lactating cows (10), but no change in steers (217). Activities of PK decreased significantly in fasting lactating cows (14), and in spontaneously ketotic cows (11). No changes were found in FBPase and G6Pase activity in spontaneously ketotic cows (13), but these two enzymes increased significantly in fasting sheep (64).

Regulation of Expression of the PEPCK Gene

To date, little research has been conducted on regulation of gene expression of enzymes of glucose metabolism in ruminants. Research for this dissertation mainly involves mRNA expression of PEPCK, and briefly of PC and FBPase in dairy cows. Therefore, selective review focusing on studies of PEPCK in nonruminants, together with a brief overview PC and FBPase, will now be presented.

The enzyme

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is one of the most extensively studied enzymes, and it was reviewed recently by Hanson and Patel (83). PEPCK catalyzes the decarboxylation of oxaloacetate to phosphoenolpyruvate, utilizing GTP as the phosphate donor. Utter and Kurahashi (201) first discovered PEPCK in 1953. There
are two forms of PEPCK in mammalian tissues, the mitochondrial (PEPCK-M) and the cytosolic form (PEPCK-C). Relative ratios of the two forms vary among species. In rats, mice, and hamsters, 90% of the hepatic PEPCK is found in the cytosol. In the liver of adult birds, all PEPCK activity is PEPCK-M. In humans and dogs, half of the total PEPCK activity is located in the cytosol and half in mitochondria, respectively. In general, the relative proportions of PEPCK-C and PEPCK-M in an animal are the same in all tissues. In chickens where PEPCK-C is absent in the liver of adult birds, however, it represents about half of the total PEPCK activity in the kidney (190). In sheep, 70% of the total PEPCK activity was found in cytosol and 30% was in mitochondria (198).

Wiese et al. (210) presented data on the intracellular distribution and/or activity of PEPCK isozymes in several tissues from rabbits, guinea pigs, rats, chickens, and pigeons. High PEPCK activities are found in liver and kidney of all species, in brown adipose tissue of rats, and in gastrointestinal mucosa of rabbits. Low activities of PEPCK are found in skeletal muscle of all species that were tested, and PEPCK was essentially undetected in brain, spleen, lung, and heart of all species. High PEPCK activity also was found in mammary tissue during lactation (101). PEPCK-M has a long half-life of about 60 h and is constitutively expressed, whereas PEPCK-C from rat liver, whose concentrations are regulated acutely by diet and hormones, has a half-life of about 8 h (94). PEPCK-C in liver and kidney is known to be important in gluconeogenesis, while the physiological role of PEPCK-M is uncertain (210). PEPCK in liver and adipose tissues was suggested to be involved in glyceroneogenesis for reesterification of free fatty acids during starvation (15). The roles of PEPCK in other tissues are not defined.
The gene for PEPCK-C from rats has 10 exons and 9 introns with a total length of 6.0 kb, which encodes 621 amino acids contained in PEPCK-C with a MW of 69,289 KDa (22). In general, the species variants of PEPCK-C have considerable sequence identity. PEPCK-C and PEPCK-M from chickens are encoded by separate nuclear genes of very different sizes, and their cDNAs have about 60% sequence identity. PEPCK-M consists of 607 amino acids (207). The cDNA for PEPCK-M from human liver has been cloned recently (137). Overall, it had a 68% DNA sequence and 70% deduced amino acid sequence identity with the human PEPCK-C cDNA. It also had an opening frame of 1920 bp, which is 54 bp longer than that of human or rat PEPCK-C cDNA. However, the PEPCK-M mRNA is 2.25 kb long, which is about 0.6 kb shorter than mRNA of PEPCK-C.

A notable property of PEPCK is the requirement for thiol reagents such as β-mercaptoethanol or dithiothreitol for maintaining the active form of the isolated enzyme. PEPCK utilizes a divalent metal ion bound in the activation site of the enzyme as an activator, and a second divalent cation, Mg$^{2+}$, is associated with GTP, the substrate (6). In general, the concentration of critical substrates such as oxaloacetate and GTP, as well as the availability of metal ions are thought to be important factors in determining the short-term regulation of PEPCK, but to date, PEPCK has no known allosteric regulators. Therefore, the regulation of PEPCK activity occurs primarily at the transcriptional level (76,110,181) and/or mRNA stability (92).
**General information about gene expression**

The half-life for PEPCK-C mRNA in rats is approximately 30 min. so that changes in the transcription rate of the gene have a marked effect on the rate of enzyme synthesis (140). It is well known that a number of factors, including diet, hormones, changes in acid/base status, and specific chemical compounds, can alter concentrations of PEPCK-C by changing its rate of synthesis. Most studies were done by using primary cell cultures, hepatoma cell lines, or liver, kidney, and adipose tissues of rats. Table 1 presents a list of factors that alter concentrations of PEPCK-C together with their known sites of action on expression of the PEPCK-C gene. It is unlikely that such diverse effectors have independent sites of action on expression of PEPCK-C. Numerous factors often act indirectly, and it seems likely that they interact at one or more of the critical regulation sites of the PEPCK-C gene promoter or on mRNA stability.

The known control mechanisms will be reviewed in detail later. The control patterns for PEPCK-C in various tissues are similar, but there is a different rate of response to these factors, and some effects may be tissue-specific. One exception is the effect of glucocorticoids, which increase the activity of hepatic PEPCK-C but decrease the synthesis rate of the enzyme in adipose tissue (133) and in 3T3-F442A adipose cells (67).

**Mechanisms of transcription regulation**

The nutritional and hormonal transcriptional regulation of the PEPCK-C promoter is a very complex process, and only known mechanisms elucidated from some key studies are reviewed here. Several approaches have been used to study these mechanisms. Introduction
Table 1. Factors that alter concentrations of PEPCK-C in mammalian tissues

<table>
<thead>
<tr>
<th>Factors</th>
<th>Tissue</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increase Concentrations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucagon (cAMP)</td>
<td>liver, adipose tissue</td>
<td>transcription, mRNA stabilization</td>
</tr>
<tr>
<td></td>
<td>kidney (less effective than in liver)</td>
<td>transcription, mRNA stabilization</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>liver, kidney</td>
<td>transcription, mRNA stabilization</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>liver</td>
<td>transcription</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>liver, adipose tissue</td>
<td>transcription, enzyme synthesis</td>
</tr>
<tr>
<td>Prolactin</td>
<td>liver, mammary gland</td>
<td>transcription, enzyme synthesis</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>kidney</td>
<td>transcription, mRNA stabilization</td>
</tr>
<tr>
<td>Glucose-free diet or fasting</td>
<td>liver</td>
<td>transcription</td>
</tr>
<tr>
<td>High-density cell culture</td>
<td>H4IIC3 hepatoma cells</td>
<td>transcription</td>
</tr>
<tr>
<td>Diabetes</td>
<td>liver, kidney, adipose tissue</td>
<td>transcription</td>
</tr>
<tr>
<td>Serotonin</td>
<td>liver, kidney, small intestine</td>
<td>mRNA</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>hepatocytes</td>
<td>transcription</td>
</tr>
<tr>
<td>Vanadate (low concentration)</td>
<td>ob/ob mice (low PEPCK activity), H35 hepatoma cells</td>
<td>mRNA</td>
</tr>
<tr>
<td>Leptin</td>
<td>H4IIC3 hepatoma cells</td>
<td>mRNA</td>
</tr>
<tr>
<td>fatty acids</td>
<td>3T3-H442A adipocyte</td>
<td>transcription</td>
</tr>
<tr>
<td><strong>Decrease concentrations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>liver, adipose tissue, kidney</td>
<td>transcription</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>adipose</td>
<td>transcription</td>
</tr>
<tr>
<td>High dietary glucose</td>
<td>liver, adipose tissue</td>
<td>transcription</td>
</tr>
<tr>
<td>Glucose</td>
<td>H4IIEC3 hepatoma cells</td>
<td>mRNA</td>
</tr>
<tr>
<td>Vanadate (high concentration)</td>
<td>hepatoma cells</td>
<td>transcription</td>
</tr>
<tr>
<td>Lithium</td>
<td>liver</td>
<td>transcription</td>
</tr>
<tr>
<td>Phorbol ester</td>
<td>hepatoma cells</td>
<td>transcription</td>
</tr>
<tr>
<td>Ochratoxin</td>
<td>kidney</td>
<td>mRNA</td>
</tr>
<tr>
<td>Liver regeneration</td>
<td>liver</td>
<td>transcription</td>
</tr>
<tr>
<td>Redox state</td>
<td>liver</td>
<td>transcription</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>kidney</td>
<td>transcription</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>hepatocytes</td>
<td>mRNA</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>hepatoma cells</td>
<td>mRNA</td>
</tr>
<tr>
<td>Inositol phosphoglycan</td>
<td>hepatocytes</td>
<td>mRNA</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>liver</td>
<td>transcription, mRNA stabilization</td>
</tr>
<tr>
<td>Age</td>
<td>hepatocytes, liver</td>
<td>transcription</td>
</tr>
</tbody>
</table>

1 Modified from Hanson et al. (83), p. 228.
of chimeric genes which contain the PEPCK promoter linked to a reporter gene, into cells in culture was used to determine the sequences responsible for control of transcription (158, 123, 191, 214). Transgenic mice, into which PEPCK-bGH (bovine growth hormone) genes were introduced, were generated to study tissue and developmental-specific regulation of the gene (131). A number of protein binding domains in the PEPCK promoter have been identified by DNase I footprinting analysis (169, 191) and gel mobility shift analysis (116). In these studies, specific block mutations and site-directed mutagenesis were introduced into the PEPCK promoter to determine functions of specific genetic elements. The majority of regulatory sites are located in a region between -500 to +73 of the PEPCK-C promoter. However, Ip et al. (98) have identified an enhancer element in the PEPCK-C promoter at -4800, which is involved in the high level of tissue-specific expression of the PEPCK-C gene in the liver. This element binds a nuclear protein, hepatic nuclear factor-3. Table 2 presents a summary of the current information on the regulatory elements contained in the PEPCK-C promoter, together with the transcriptional factor(s) that bind to these elements.

cAMP. As shown in Table 2, several cis-acting sequences within the PEPCK-C promoter mediate cAMP activity. The presence of a cAMP regulatory element (CRE) in the PEPCK-C gene was first suggested by Short et al. (191). The consensus sequence, TTACGTCA, which maps between -90 and -83 of the PEPCK-C promoter, was identified as CRE1 by Boker et al. (28). PEPCK-C promoter also contains the CRE2 site, which has a C to G substitution at -87 of CRE1. The upstream CRE weakly binds nuclear protein extracted from the liver (169). The CRE of PEPCK-C acts in concert with upstream elements that regulate transcription of the gene by cAMP. A block mutation in either the
Table 2. Summary of regulatory domains in the PEPCK-C promoter

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Location</th>
<th>Sequence</th>
<th>Binding protein</th>
<th>Function in transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATA</td>
<td>-29/-23</td>
<td>TATTTAAA</td>
<td>TBP</td>
<td>basal</td>
</tr>
<tr>
<td>CRE-1</td>
<td>-91/-84</td>
<td>TTACGTCA</td>
<td>C/EBPβ, CREB, Fos/Jun, DBP</td>
<td>basal, transcription/cAMP</td>
</tr>
<tr>
<td>P1</td>
<td>-116/-104</td>
<td>TGGCTATGACCA</td>
<td>NF-1/CTF</td>
<td>basal</td>
</tr>
<tr>
<td>CRE-2</td>
<td>-144/-137</td>
<td>TTAGGTCA</td>
<td>C/EBP</td>
<td>cAMP</td>
</tr>
<tr>
<td>P2</td>
<td>-190/-185</td>
<td>ATTAAC</td>
<td>HNF-1</td>
<td>renal expression</td>
</tr>
<tr>
<td>P3(I)</td>
<td>-243/-235</td>
<td>TTGTGTAAG</td>
<td>C/EBPβ, DBP</td>
<td>basal expression/cAMP</td>
</tr>
<tr>
<td>P3(II)</td>
<td>-258/-252</td>
<td>TTAGTCA</td>
<td>Fos/Jun</td>
<td>basal, cAMP</td>
</tr>
<tr>
<td>P4</td>
<td>-282/-274</td>
<td>ATCAGCAAC</td>
<td>C/EBPβ</td>
<td>basal, cAMP</td>
</tr>
<tr>
<td>TRE</td>
<td>-332/-316</td>
<td>TGGGGGTCAAG</td>
<td>TR</td>
<td>thyroid hormone</td>
</tr>
<tr>
<td>GR2</td>
<td>-363/-353</td>
<td>CATATGAAGTC</td>
<td>GR</td>
<td>glucocorticoids</td>
</tr>
<tr>
<td>P5/G1R1</td>
<td>-385/-374</td>
<td>ACACAAAAATGTG</td>
<td>GR</td>
<td>glucocorticoids</td>
</tr>
<tr>
<td>IRE/AF2</td>
<td>-41/-400</td>
<td>TGGTGTGTGACA</td>
<td>unknown</td>
<td>insulins, glucocorticoids</td>
</tr>
<tr>
<td>P6/AF1</td>
<td>-450/-436</td>
<td>ATGACCTTTGGC</td>
<td>unknown</td>
<td>glucocorticoids</td>
</tr>
</tbody>
</table>

* Taken from Hanson et al. (83), p. 235.

CRE1 or P3(I) decreases transcription by 70%, but simultaneous mutation of both elements completely eliminates transcriptional activation of PEPCK-C promoter by cAMP (116). To date, only members of the leucine zipper family of transcriptional factors [i.e., cAMP regulatory element binding protein (CREB), CAAT/enhancer binding protein (C/EBP), D-binding protein (DBP), and Fos/Jun dimers] are known to bind to CRE and to induce transcription from the PEPCK-C promoter when their genes are cotransfected and expressed in hepatoma cells (146, 170). Recently, Roesler et al. (171) demonstrated that C/EBPα is
required for mediating cAMP responsiveness of the PEPCK promoter, although the DNA binding domain of C/EBPα was not required for this activity in hepatoma cells.

**Insulin.** Insulin has been suggested by Granner et al. as the "dominant negative regulator" of PEPCK-C gene expression because of its ability to inhibit transcription of the PEPCK gene in hepatoma cells in the presence of cAMP and glucocorticoids (181) and to decrease the basal level of transcription of the gene (76). Despite the strong effects of insulin on PEPCK-C gene transcription, delineating the insulin response elements in the PEPCK-C gene has been elusive. The activity of insulin to block the inductive effect of cAMP on transcription from the PEPCK-C promoter in cells transfected with chimeric genes, in most cases, has not been demonstrated (214). Finally, an insulin regulatory sequence (IRS). TGGTGTTTTTG, between -416 and -407 of the PEPCK-C promoter has been described by O'Brien et al. (143). They reported that a mutation in the core IRS sequence of the PEPCK promoter resulted in approximately 50% inhibition of the ability of insulin to block expression of the PEPCK-CAT gene in the presence of both cAMP and glucocorticoids. Because of the dominant role of insulin, it is possible that there are additional elements within the PEPCK-C promoter or other mechanisms involved in the transcriptional effect of insulin. By similar approaches, O'Brien et al. (142) found that phorbol ester also inhibits PEPCK-C gene transcription through the IRS as does insulin.

By using in vitro run-on assays with specific probes directed against regions on the PEPCK-C gene, Sasaki and Granner (180) analyzed whether transcription proceeds uniformly across this gene in response to insulin and cAMP. The primary transcript of PEPCK was synthesized at a rate of about 2500 nucleotides per minute in the presence of
cAMP but decreased to 1000 nucleotides per minute when insulin was present. These results suggested that both insulin and cAMP exert their primary effects directly at the level of transcriptional initiation, but in opposite directions. Sutherland et al. (197) recently found that wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), blocks the inhibition by insulin of glucocorticoid- and cAMP-stimulated PEPCK-C gene transcription. In contrast, wortmannin does not block the similar effects of phorbol esters. Gabbay et al. (70) demonstrated that neither insulin nor phorbol ester regulation of PEPCK-C gene transcription requires activation of the Ras/MAP kinase pathway. These studies suggested that PI3K is required for the regulation of PEPCK gene transcription by insulin but not by phorbol esters.

*Glucocorticoids.* Glucocorticoids have long been thought to have a permissive action on gluconeogenesis. The additive effect of glucocorticoids on PEPCK gene expression was first demonstrated in 1982 by Salavert and Lynedjian (174), who reported that glucagon had only marginal stimulatory effects on the synthesis of PEPCK-C in hepatocytes in the absence of dexamethasone. Two regions of the PEPCK-C promoter: one distal between -1264 and -1111 and the other more proximal between -468 and -420 (glucocorticoid response unit, GRU) have been implicated in the regulation of PEPCK gene transcription by glucocorticoids (97). GRU contains two glucocorticoid receptor-binding sites (GR1 and GR2) and two accessory factors (AF1 and AF2). Interestingly, IRS is found within the AF2 site. Recently, Imai et al. (96) demonstrated that maximal induction of transcription from the PEPCK-C promoter by glucocorticoids required both the CRE and the GRU; they also noted an interaction between the glucocorticoid receptor and CREB, suggesting that these proteins
could be involved in the control of PEPCK gene transcription. This also could explain the additive effects of cAMP and glucocorticoids in stimulating PEPCK gene transcription.

*Diabetes and diet.* The use of PEPCK-bGH transgenic mice provided further information on dietary and hormonal regulation of PEPCK gene expression, as well as information on PEPCK gene expression in diabetes. PEPCK activity (47), protein (183), and mRNA (186) are all increased in animal models of noninsulin dependent diabetes mellitus (NIDDM) and streptozotocin-induced diabetes. An analysis of the regulatory elements in the PEPCK-C promoter involved in the response of the PEPCK-C gene to diabetes has been carried out by using transgenic mice with a deletion in the GRU region of the PEPCK-C promoter and a mutation in the CRE and P3(I) regions (66). Removal of GRU eliminated the induction of transcription of the PEPCK-bGH gene in the liver of diabetic mice, and adrenalectomy of diabetic mice also eliminated the induction of hepatic PEPCK-C mRNA. Mutation of CREs of the PEPCK promoter did not limit induction of PEPCK transcription by diabetes. These results suggested an important role for glucocorticoids in the regulation of PEPCK-C gene transcription during diabetes.

Acute and marked dietary regulation of PEPCK gene transcription is another important feature of the PEPCK-C gene (110, 131). Dietary regulations are usually thought to be mediated via changes in concentrations of glucagon (via cAMP) and insulin in plasma. Surprisingly, studies using transgenic mice have shown that this is not the case. Patel et al. (147) found that removal of both CRE1 and P(1) domains, either singly or in combination, did not inhibit the induction of PEPCK-bGH gene expression in the liver of transgenic mice fed a carbohydrate-free diet. These results indicated that conventional cAMP regulatory
elements are not directly involved in the induction of PEPCK-C gene transcription caused by carbohydrate-free diets or diabetes. IRS is also not involved in negative effects of carbohydrate feeding on PEPCK gene transcription because its deletion did not prevent the inhibition of expression of PEPCK-bGH gene in the liver of transgenic mice fed a high-carbohydrate diet (66). Davies et al. (51) recently showed that concentrations of phosphorylated CREB were decreased significantly in the liver of diabetic rats and that insulin treatment reversed this effect. That study also showed that overexpression of PEPCK is not linked to the cAMP signaling system. The PEPCK-C gene promoter in patients with NIDDM was examined recently, and no DNA sequence polymorphisms were found in any patient (120). This suggests that mutations in cis-acting PEPCK gene regulatory elements do not constitute a common cause of NIDDM.

**PC and FBPase**

In contrast to PEPCK, hormonal and nutritional regulations of the PC and FBPase genes are poorly understood. Pyruvate carboxylase (EC 6.4.1.1.) is a biotin-containing enzyme that catalyses the carboxylation of pyruvate to form oxaloacetate for gluconeogenesis and replenishing intermediates of TCA cycle for fatty acid, amino acid, and neurotransmitter synthesis. The native enzyme consists of four identical subunits arranged in a tetrahedron-like structure. Mammalian PC is regulated allosterically by acetyl-CoA, which stimulates the cleavage of ATP in the first partial reaction and induces a conformational change in the tetrameric structure of the enzyme (7). Recently, cDNA for PC has been cloned and the deduced amino acid sequence has been determined for mice, rats, and humans (100, 121.
There is a high degree of amino acid identity among these species, and the size of mature PC mRNA is 4.2 kb in all three species. Northern blotting analysis showed that PC mRNA is highly expressed in liver, kidney, adipose tissues, and brain; moderately expressed in heart, adrenal gland, and lactating mammary gland; and expressed at a low level in spleen and skeletal muscle in rats (100). Few studies have been done on hormonal regulation of the PC gene. One study with 3T3-L1 cells showed that both PC activity and PC mRNA were decreased markedly by cAMP alone or together with insulin in these cells, but the amount of PC protein was not changed (220). In contrast to being exclusively a mitochondrial enzyme in most species, PC in ruminants is found in both cytosol and mitochondria. Acetyl-CoA, propionyl-CoA, and butyryl-CoA are all activators of PC in ruminants (17).

Fructose 1,6-bisphosphatase (EC 3.1.3.11) catalyzes the hydrolysis of Fru-2,6-P₂ to fructose 6-phosphate and inorganic phosphate in gluconeogenesis. The enzyme, a tetramer of four identical polypeptide chains, is subjected to both acute and long-term hormonal regulation. The activity of FBPase is regulated allosterically by AMP and by Fru-2,6-P₂ which is a potent inhibitor of FBPase. Fru-2,6-P₂ has been postulated to bind either the active site (153) or a separate allosteric site (204). Concentrations of Fru-2,6-P₂ are strictly regulated by PFK-2/FBPase-2, which is regulated by phosphorylation/dephosphorylation and by gene expression of hormonal control (49, 154, 172).

The cDNA for FBPase has been cloned from pigs, rats, and humans, and the primary sequence of deduced amino acids of the enzyme is highly homologous among these species (59, 211). FBPase is also under long-term regulation by change in concentrations of the enzyme, which increases in livers from diabetic and fasted rats. FBPase mRNA was
increased 10-fold in the liver from diabetic rats and decreased to control levels after 24 h of insulin treatment, but the decreases in FBPase mRNA upon insulin treatment were slower relative to PEPCK mRNA (59). Also, FBPase mRNA in primary rat hepatocyte cultures was increased by cAMP and decreased by insulin (60). By using Northern blotting with a rat liver cDNA, FBPase mRNA was detected only in the liver and kidney of rats (59), although significant activities of FBPase existed in brain and muscle. The size of rat FBPase mRNA was about 1.4 kb of single species, and its half life was 5 to 6 h (60).

**Glucagon in Ruminants**

*History and metabolic effects of glucagon*

Glucagon was discovered in 1923 after Kimball and Marlin succeeded in separating the hyperglycemic factor from the hypoglycemic component of the crude extracts of canine pancreatic tissue (107). Glucagon was purified in crystalline form by Staub et al. in 1955 (194), and Unger developed a radioimmunoassay for glucagon that made it possible to measure its concentration in the plasma of dogs and humans (200).

The preproglucagon gene, located on the long arm of chromosome two, is expressed in pancreatic α-cells, small intestinal L-cells, and certain hypothalamic cells. These cells produce different kinds of glucagon-like polypeptides. Only the pancreatic α-cells have the enzymes to cleave proglucagon to "true glucagon", a 29 amino acid polypeptide. The amino acid sequence of glucagon is the same in all mammals studied, except the guinea pig, so glucagon exhibits a high degree of conservation.
Metabolic effects of glucagon have been studied extensively in dogs and humans by using various infusion techniques, i.e., pharmacological, supraphysiological, or physiological doses of glucagon together with somatostain clamps or diabetic models. A vital physiological role of glucagon is increasing hepatic glucose production for maintaining glucose homeostasis. Glucagon increases both glycogenolysis and gluconeogenesis in nonruminants. The effect on glycogenolysis is time-dependent, i.e., initial and transitory, but the effect on gluconeogenesis is time-independent (48). Glucagon increases gluconeogenesis via promoting hepatic uptake of amino acids and increasing activities of key gluconeogenic enzymes. Decreases in concentrations of plasma amino acids are common during hyperglucagonemia. Contributions of glucagon to postabsorptive glucose production can be confirmed by glucagon immunoneutralization by using a high-affinity monoclonal glucagon antibody. Plasma glucose was decreased in normal rabbits and decreased even more in alloxan-induced diabetic rabbits by glucagon immunoneutralization (29).

Both in dogs and humans, glucagon increases lipolysis in adipose tissue (111), especially when plasma insulin concentration is controlled to be normal or during moderate hyperglucagonemia in humans (45). During short-term hyperglucagonemia, degradation of protein was not changed (65); yet, it is not known whether protein degradation increases during long-term hyperglucagonemia.

The known metabolic effects of glucagon in ruminants were evaluated primarily by Brockman et al. (32, 43). Glucagon was clearly shown to increase hepatic glucose production and gluconeogenesis in sheep by arterio-venous catheterization and tracer techniques. Glucagon increased hepatic uptake of alanine, glutamine, other amino acids, and
lactate, but not glycerol (43). Also, Brockman (37) showed that utilization of propionate seemed not to be regulated by insulin or glucagon. However, other in vitro studies indicated that glucagon may enhance the conversion of propionate to glucose (50, 182).

Infusion of glucagon has increased plasma glycerol when glucagon-stimulated insulin release was prevented, an effect that implies that glucagon increases lipolysis (33). In normal sheep, however, hyperinsulinemia prevented this increase, and concentrations of plasma NEFA actually decreased during infusions of glucagon (19). Further, glucagon showed no lipolytic effects in vitro in studies of adipose tissue from sheep and dairy steers (63). Therefore, the presence of a lipolytic effect of glucagon is still questionable in ruminants. To date, few known studies have been done on glucagon effects in dairy cows.

**Control of glucagon secretion**

Release of glucagon from the pancreas varies depending on nutritional and physiological status, stress, and pathological status. Glucagon secretion is controlled by nutrients and metabolites, hormones, and the autonomic nervous system. Extensive research has been done on glucagon release in nonruminants, but the current review mainly summarizes studies in ruminants.

*Glucose.* Infusion of a 20% glucose solution into the duodenum of steers caused a decrease in plasma glucagon concentrations (25). Intravenous infusion of glucose into normal sheep also decreased glucagon concentrations (21, 34, 39). In dairy cows, 20 min of intrajugular glucose infusion cause a significant decrease of plasma glucagon concentrations (178). Low glucose concentrations resulting from starvation, alcohol infusion, insulin
administration, or blockage of glucose uptake by 2-deoxyglucose stimulate the \( \alpha \)-cells to release glucagon. Bloom et al. (27) have shown that injection of 2-deoxyglucose into calves could increase glucagon concentrations.

**Amino acids.** Amino acids stimulate the release of glucagon. The gluconeogenic amino acid, alanine, is a strong \( \alpha \)-cell secretagogue, but so are certain nongluconeogenic amino acids such as arginine (139, 144). Abomasal infusion of protein into sheep (18) and duodenal infusion of amino acids into steers (26) caused an increase in plasma glucagon concentration. Serine, lysine, aspargine, alanine, and glycine stimulated glucagon release by pancreatic slices from lambs, with glycine and alanine being the most stimulating amino acids (20). Also, intravenous infusion of 17 amino acids in sheep, each at a dose of 3 mmol/kg over 30 min. indicated that alanine, glycine, and serine induced a greater enhancement of both glucagon and insulin secretion than did other amino acids (109). Leucine was the most effective amino acid in stimulating insulin secretion, but it did not increase in glucagon and growth hormone secretion. No amino acid was able to specifically stimulate glucagon secretion without also increasing secretion of insulin or growth hormone (109). Amino acid release during protein digestion probably accounts for the glucagon increment that occurred in sheep after feeding (21) and in grain-fed steers (26). Because a high glucagon concentration causes a decrease in plasma amino acids (127), the relationship between amino acids and glucagon may reflect a physiological feedback.

**Volatile fatty acids (VFA).** In ruminants, VFA from carbohydrate fermentation in the rumen are the most important energy sources for the body, and research suggests that VFA play a role in the control of glucagon secretion. Earlier studies have shown that
supraphysiological doses of VFA stimulate the release of insulin and glucagon from the pancreas (21, 53, 95, 125). However, the effects of physiological levels of VFA on release of these hormones have been controversial (53, 196). Physiological infusions of butyrate, but not propionate, increased glucagon concentration (38). However, other studies have shown that infusion of propionate at a physiological rate through either a mesenteric vein or a femoral vein increased concentrations of plasma insulin and glucagon (150, 176, 177). Mineo et al. (135) did a study to investigate chemical specificity of VFA in stimulating insulin and glucagon secretion in sheep. N-butyric acid was the most effective for both insulin and glucagon secretion among the VFA tested. Long-chain free fatty acids were associated with a decreased glucagon concentration during fasting in sheep (46).

**Insulin.** In sheep, insulin infusion stimulates glucagon secretion, but effects seem to be mediated via hypoglycemia because simultaneous infusion of glucose negated any increases in glucagon (34). Actually, the relationship between insulin and the role of plasma insulin and/or glucose in the control of glucagon secretion has not been elucidated clearly. Because hyperglucagonemia is a hallmark of both Type I and Type II diabetes in humans, extensive studies have been done to investigate the role of insulin and glucose in regulating glucagon secretion. Since Samols et al. (175) discovered in 1965 that insulin was a powerful inhibitor of glucagon secretion, several in vitro and in vivo studies have supported their conclusion. A study that used isolated α-cells, however, showed that insulin was unable to affect glucagon secretion (155). An in vivo study in alloxan-induced diabetic dogs failed to demonstrate a direct role of insulin in controlling glucagon secretion (78). A recent study also showed that plasma glucagon concentration did not change in hyperinsulinemic
euglycemic rats (122). Moreover, expression of the glucagon gene was insensitive to changes in concentrations of plasma glucose and insulin (122). In contrast to the controversial effects of insulin on glucagon, glucagon definitely has a stimulatory effect on insulin secretion (175). This stimulation may be achieved indirectly by glucagon increasing glucose production, which in turn increases insulin secretion. On the other hand, Ohenda found that C-terminal peptides of glucagon enhanced insulin release and that glucagon peptide 21-29 is the minimal structure among the C-terminal glucagon fragments to stimulate insulin secretion (145).

*Gastrointestinal hormones and the autonomic nervous system.* The use of phentolamine in exercised sheep was associated with an increase in insulin concentration and seemed to delay the exercise-induced increase in glucagon. Also, administration of propranolol had no effect on glucose turnover or on concentrations of plasma glucagon and insulin. These results suggested α-adrenergic mediation of the sympathetic influence on insulin and glucagon secretion (35, 36, 41, 42). Epinephrine stimulates glucagon secretion and inhibits insulin secretion (19), which suggests a role for these hormones during stress.

*Physiological and pathological states and stress.* Bassett et al. (21) has shown that glucagon concentration peaks 2 to 4 hours after feeding, but a postprandial increase is not always observed (17). Glucagon concentrations usually decreased during fasting (21, 46), but they sometimes remained unchanged (75). Plasma glucagon concentrations were significantly greater in sheep given a low-energy diet compared with those given a high-energy diet (141). However, in prepartal cows, there were small variations and no influence of feeding on glucagon levels, but there was a strong increase after calving, especially in the
cows fed the highest amounts of concentrates (93). Glucagon concentrations were similar throughout lactation, and above average milk production was associated with greater glucagon, less insulin, and lower concentrations of plasma glucose (85). Other studies, however, showed opposite results. Glucagon concentrations tended to be greater in low-producing cows than in high-producing cows (21) and tended to increase as milk production decreased (84). Glucagon concentration tended to increase (124) or to be greater in early lactation (148).

Plasma glucagon concentrations in ketotic cows also have not been consistent. Hyperketonemic cows had lower insulin and higher glucagon concentrations than normal cows (93). Sasaki et al. (179) also reported that blood glucagon increased significantly in spontaneously ketotic cows. Simultaneous injections of insulin and glucose caused plasma glucagon to decrease and insulin to increase, which resulted in a more efficient treatment of ketosis than injection of glucose alone (179). However, in experimentally ketotic cows, deBoer et al. (52) found that plasma glucagon concentrations decreased from 3 wk postpartum to the experimentally induced ketonemic period. A similar decrease was observed in spontaneously ketotic cows, although secondarily ketotic cows had increased concentrations of glucagon (25).

Summary. Although some information exists, results of studies on glucagon levels in fed and fasted states as a function of energy level of the feed, stage of lactation, and ketosis are not consistent. Because digestion and absorption in adult ruminants are prolonged when compared with nonruminants and because the feeding and management system is less consistent in ruminants, the effects of dietary influences and diurnal patterns on glucagon
levels are more complex. Another important reason for inconsistent results is that there have been some methodological difficulties in the measurement of plasma glucagon by radioimmunoassay because few antisera were specific for pancreatic glucagon.

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METABOLIC RESPONSES OF NORMAL LACTATING DAIRY COWS TO 14-DAY INTRAVENOUS INFUSIONS OF GLUCAGON

A paper submitted to the Journal of Dairy Science


ABSTRACT

Twenty cows were assigned at parturition into two groups to study metabolic effects of continuous infusions of glucagon. Groups were normal controls and normal cows treated with glucagon at 10 mg/d for 14 d starting at 21 d postpartum. Daily blood samples and nine periodic liver biopsies were taken from d 7 to d 49 postpartum. Plasma glucagon increased 6- to 7-fold during infusions into treated cows. Plasma insulin increased during wk 1 of glucagon infusions. Plasma glucose increased by 11.5 and 9.0 mg/dl during wk 1 and wk 2 of glucagon infusions. Neither plasma NEFA, BHBA, nor urea N were affected by glucagon infusions. Liver glycogen decreased by d 2 of glucagon infusions, but it was restored by d 7 of infusions and had increased to 169% of the preinfusion baseline at 3 d after cessation of glucagon infusions. Milk production was less during glucagon infusions. Both milk protein yield and milk protein percentage decreased during glucagon infusions, which implies a decreased availability of amino acids for milk protein synthesis. Feed intakes did not increase during glucagon infusions, which was in contrast to the control group. The results indicate that glucagon infusions cause a net hepatic glycogenolysis initially and enhance plasma glucose concentrations, but there is no evidence for increased lipolysis from
adipose tissue.

(Key words: dairy cows, glucagon, metabolism)

Abbreviation key: BCS = body condition score. NC = normal control cows. NTG = normal cows treated with glucagon. TAG = triacylglycerols.

INTRODUCTION

Glucagon has broad metabolic effects on glucose, lipid, and amino acid metabolism both in vivo and in vitro as described in the literature. The major target organ of glucagon, however, is the liver where it stimulates both glycogenolysis and gluconeogenesis, thereby increasing hepatic glucose output (11, 12). Glucagon antagonizes actions of insulin in the liver and plays a major role, together with insulin, in regulation of glucose homeostasis for several physiological and nutritional conditions in nonruminants (26). By using various infusion techniques, such as a somatostatin clamp, the physiological actions of glucagon in vivo have been studied extensively both in humans and in dogs (11, 12, 20).

Ruminants are in a continuous state of gluconeogenesis even when they are in their fed state, which differs greatly from nonruminants (2). The mechanisms for dietary and hormonal controls of glucose metabolism in ruminants are not thoroughly clarified; however, some studies have shown that hormones such as glucagon, insulin, growth hormone, glucocorticoids, and catecholamines play important roles in regulating glucose metabolism (23, 24, 31). In sheep, short-term administration of glucagon increased the output of hepatic glucose and caused hyperglycemia, which were both exaggerated in insulin-controlled, alloxan-diabetic sheep (3, 7). Infusion of glucagon increased plasma glycerol when
glucagon-stimulated insulin release was prevented, an effect implying that glucagon increases lipolysis (7). In normal sheep, however, hyperinsulinemia prevented this increase, and concentrations of plasma NEFA actually decreased during infusions of glucagon (2). Further, glucagon showed no lipolytic effects in vitro in studies of adipose tissue from sheep and dairy steers (19). Therefore, the presence of a lipolytic effect of glucagon is still questionable in ruminants.

In dairy cows, ketosis is a major metabolic disease that is characterized by hypoglycemia, depletion of liver glycogen, hyperketonemia, and fatty liver. Fatty liver seems to precede the occurrence of clinical ketosis, and ratios of liver triacylglycerols (TAG) to glycogen greater than 2.5 seem to be required for clinical ketosis to occur (17). Ketosis also is thought to generally result from inadequate availability of endogenous glucose (1). Our hypothesis was that exogenous glucagon infused into dairy cows would increase the availability of endogenous glucose without also increasing the availability of endogenous NEFA mobilized from adipose tissue. That hypothesis is part of a broader hypothesis that pharmacological infusions of glucagon would decrease development of fatty liver and clinical ketosis in lactating dairy cows by increasing availability of glucose and increasing the ability of the liver to resist excess deposition of TAG. Therefore, our objective was to determine some of the pharmacological effects of long-term intravenous infusions of glucagon in normal, early-lactating dairy cows with an emphasis on metabolic regulation of glucose metabolism in a global manner.
MATERIALS AND METHODS

Experimental Design, Treatment, and Management of Cows

Twenty normal multiparous Holstein cows with an average body condition score (BSC) of approximately 3.6 at 15 d prepartum were used. Cows were selected on the basis of having a ratio of liver TAG to glycogen (wt/wt) of less than 1.5 at 6 DIM. Pairs of cows calving in the same 2-wk interval were considered a block and randomly assigned to a group of either normal control (NC) cows infused with 0.15 NaCl or a group of normal cows treated with glucagon (NTG). This design provided 10 blocks or replications of the two treatments.

For NTG cows, a subcutaneous polyethylene catheter was implanted into each jugular vein at 20 DIM, one day before infusions of glucagon were to begin. A catheter was implanted into only one jugular vein for NC cows. Lyophilized bovine glucagon (provided by Dr. Ronald Chance, Eli Lilly & Co., Indianapolis, IN) was dissolved in 0.15 M NaCl at pH 10.25 with a final concentration of 20.833 μg/ml. All glassware, pipettes, infusate containers, and infusion tubing in contact with glucagon solutions were rinsed before use with a 1% solution of enzyme-free BSA. Quantitative infusions into the NTG group and NC group were done continuously from 21 to 35 DIM at a rate of 20 ml/h of either glucagon (10 mg/d) or 0.15 M NaCl, respectively, via one catheter by using volumetric pumps (INFUCHECK™ model 1500, IVAC® Corporation, San Diego, CA). At the end of each 14-d infusion, the infusion rate was decreased by 2 ml/h every hour from 10:00 to 19:00 h to avoid abrupt withdrawal of glucagon.
All cows were fed a TMR and grass hay during the prepartum period. The TMR (DM basis) consisted of 41% corn silage, 15.4% alfalfa hay, 10.3% corn gluten feed, 10.3% oat silage, and 23% concentrate containing 72% rolled corn, 24% soybean meal, 1.1% Na₂CO₃, 1.1% (Ca₃(PO₄)₂, 0.6% MgO, 0.6% NaCl, 0.3% CaCO₃, and 0.3% X-Cel Ruminant Trace Mineral Premix ®. After calving, cows were fed ad libitum with 70% TMR, 5% whole cottonseeds, and 25% commercial supplement that contained 58% soy hulls, 24% soyPLUS, 6.8% fish meal, 6.8% white grease, and 3.8% minerals. Diets were balanced according to NRC recommendations. Daily feed intakes were measured for each cow from 7 to 49 DIM. Three evaluators determined BSC at -30, -15, 1, 14, 34, and 49 DIM (18). Cows were housed in a tie-stall barn, milked three times daily, and weighed weekly from 1 to 49 DIM. Milk production was recorded daily. Milk samples were collected at 13, 20, 23, 34, 37, and 49 DIM and analyzed for percentages of fat, protein, and lactose by mid-infrared spectrophotometry (Milk-O-Scan 203, Foss Food Technology, Eden Prairie, MN). All cows were handled and treated in accordance with guidelines established by the Iowa State University Animal Care Committee.

Sampling Procedures

Puncture biopsies of 4 to 6 g of liver were obtained under local anesthesia from each cow on 6, 13, 20, 23, 27, 35, 38, 42, and 49 DIM. Most biopsies were done in the afternoon except the biopsies on d 35, which were done at 21:00 h, 2 h after complete cessation of infusions. Location of the liver was verified by ultrasound visualization. After a liver
sample was removed from the biopsy cannula, it was immediately placed on absorbent paper, blotted free of blood, frozen in liquid nitrogen, and stored at -80°C.

Blood samples were withdrawn daily from the coccygeal vein from 7 to 49 DIM into vacuum tubes containing Na₂-EDTA. Blood samples were collected before liver biopsies on days that biopsies were obtained. On the day an infusion was begun and on the day it was stopped, a series of blood samples were collected from the jugular catheter at short intervals. Blood samples were placed in ice until centrifugation, which was done within 2 h after samples were withdrawn. Plasma was divided into three aliquots and stored at -20°C. One ml of plasma to be used for glucagon determinations was mixed with 500 KIU of aprotinin, an inhibitor of proteolysis (Boehringer-Mannheim, Cat. No. 1583794), before freezing.

**Analytical Procedures**

Frozen liver was powdered in liquid N₂ and homogenized in 7% perchloric acid (wt/vol); then, concentrations of glycogen were determined by an iodine binding assay (16). Lipid was extracted from powdered liver with 2:1 (vol/vol) chloroform-methanol, and concentrations of total lipid were determined gravimetrically after drying under a stream of air (17), which was determined to be equivalent to drying under N₂. The lipid extract was redissolved in 3:2 (vol/vol) hexane:isopropanol, and concentrations of TAG were determined (17). The DM percentages of liver samples were determined gravimetrically by weighing before and after lyophilization. Concentrations of plasma glucagon and insulin were determined by radioimmunoassay by using commercial kits (Diagnostic Products Corporation, Los Angeles, CA). Plasma metabolites (glucose, NEFA, BHBA, urea nitrogen)
were determined by using a microcentrifugal autoanalyzer (Monarch Plus, Instrumentation Laboratory, Lexington, MA) and coupled enzymatic assays.

Statistical Analysis

The experiment was analyzed as a completely randomized block design. Data from the two treatment groups were subjected to ANOVA by using general linear models procedures of SAS (28). A separate analysis was done for each time period or sampling day to determine whether mean values for NC and NTG cows differed. Because of a few missing values, means were adjusted for block differences (LSMeans in SAS's Proc GLM). Additional analyses of time x treatment effects were done on functions of the repeated measures on the same cow, such as the differences between wk 3 and wk 4 or between wk 3 and wk 5 in NC and NTG cows. The changes by time within each treatment group was evaluated by a paired t test (29) by testing whether differences between two times differed from zero when averaged over 10 cows. Tests of significance with $P \leq 0.05$ were considered statistically significant.

RESULTS

Plasma Hormones and Metabolites

Plasma glucagon concentrations in NTG cows increased immediately after glucagon infusions began and plateaued during the entire infusion period, however, concentrations declined slightly from the beginning to the end (Figure 1A). Overall, glucagon concentrations were elevated by 6- to 7- fold during glucagon infusions when compared with
Figure 1. Concentrations of plasma glucagon and insulin in normal control cows (○) and normal cows treated with glucagon (●) from 21 to 35 DIM. Hormone concentrations shown in the figure are average values of two samples from two consecutive days around each biopsy, except values at the end of infusions were from samples 2 h after infusions. Values at the day infusions began and one day before infusions ended were from samples of only that day. Significant differences between treatments at a given time were indicated by * $P < 0.05$, or ***/ $P < 0.0001$. A) Plasma glucagon: Time x treatment effects between d 20 and d 23, $P < 0.0001$; d 20 and d 27, $P < 0.0004$; and, d 20 and d 35, $P < 0.14$. B) Plasma insulin: Time x treatment effects between d 20 and d 23, or d 35, $P > 0.05$; and, d 20 and d 27, $P < 0.02$. 
the preinfusion baseline. Plasma glucagon concentrations in NTG cows decreased immediately to slightly greater than that of NC cows after glucagon infusion ended, and they were less at d 7 than at d 49 postpartum in the NC group (P < 0.01).

Plasma insulin concentration was significantly greater at d 7 of infusions of glucagon in the NTG group than in the NC group (Figure 1B). Further, the time x treatment effect between d 20 and d 27 was significant (P < 0.02). Insulin concentrations of NTG cows decreased to that of NC cows at the end of glucagon infusions and increased thereafter. Thus, plasma insulin was increased by glucagon infusions; however, there were considerable variations in insulin values among individual cows as indicated by large values of SEM. One reason for the variations was the heterogeneous insulin responses of cows to glucagon infusions. Even in the first hours of glucagon infusions among the 10 NTG cows, six cows showed different degrees of increased plasma insulin, three cows did not change at all, and one cow had a doubtful response (data not shown). Plasma insulin concentrations were also less at d 7 than at d 49 postpartum in the NC group (P < 0.01).

Weekly averages of plasma glucose concentrations in NTG cows (Figure 2A) increased by 11.5 (22%) and 9.0 mg/dl (17%), respectively, during the two weeks of glucagon infusions (i.e., wk 4 and 5) when compared with wk 3 before infusions (P < 0.0001). Plasma glucose of NTG cows in the first hours of glucagon infusion increased by 14.4 mg/dl (27%) compared with the preinfusion baseline. It peaked 20 min after glucagon infusion began and plateaued for the remaining 3.67 h of sampling (data not shown). Concentrations of plasma glucose were elevated constantly throughout the glucagon infusion period in NTG cows. Glucose did not change in NC cows during wk 4 and 5, but it was
Figure 2. Average concentrations of plasma metabolites by week in normal control cows (■) and normal cows treated with glucagon (□) from 21 to 35 DIM. Significant differences between treatment at a given time are indicated by * \( P < 0.05 \), ** \( P < 0.01 \), or *** \( P < 0.001 \). A) Plasma glucose: Time \( \times \) treatment effects between wk 3 and wk 4, \( P < 0.004 \); wk 3 and wk 5, \( P < 0.0006 \); wk 3 and wk 6, \( P < 0.88 \); and, wk 5 and 6, \( P < 0.003 \). B) Plasma nonesterified fatty acids (NEFA). C) Plasma \( \beta \)-hydroxybutyrate (BHBA). D) Plasma urea nitrogen: Time \( \times \) treatment effects for NEFA, BHBA, and plasma urea nitrogen between wk 3 and wk 4, or wk 5, or wk 6, and, wk 5 and 6 were \( P > 0.05 \).
Glucagon

Time postpartum (wk)

A

Glucose (mg/dl)

B

NEFA (peq/L)

C

β-hydroxybutyrate (mg/dl)

D

Urea nitrogen (mg/dl)

2 3 4 5 6 7

Time postpartum (wk)
greater in wk 7 than in wk 2 ($P < 0.05$).

Plasma NEFA concentration (Figure 2B) decreased continually with lactation in both groups from wk 2 to wk 7, and, overall, it was less in NTG than in NC cows. The lower concentration of plasma NEFA was not caused by glucagon because it existed before infusions began, and time x treatment effects between preinfusion and during infusion were not present ($P > 0.05$). Therefore, plasma NEFA was influenced by stage of lactation but not by glucagon infusions.

Neither plasma BHBA nor urea nitrogen were different between groups (Figure 2C and 2D) and, therefore, they were not affected by glucagon infusions. Also, their concentrations were relatively constant throughout the experiment.

**Composition of the Liver**

Liver glycogen contents in NTG cows were changed markedly by glucagon infusions (Figure 3A). When compared with 20 DIM as a baseline, liver glycogen concentration had decreased by an actual value of 0.94 ± 0.12% ($P < 0.0001$) at d 2 of glucagon infusions, but it was completely restored to baseline at d 7. Later, it increased to 1.32 ± 0.30% ($P < 0.002$) above baseline at d 14 of glucagon infusions and increased further to 1.65 ± 0.31% ($P < 0.0005$) above baseline at 3 d after infusions ended. In contrast, there were no significant changes of liver glycogen in NC cows at any time.

Liver TAG concentrations decreased significantly with the progression of lactation in both NC and NTG cows (Figure 3B). For instance, TAG decreased by actual values of 2.5 ± 0.63% in NC cows ($P < 0.004$) and 3.0 ± 0.76% in NTG cows ($P < 0.003$) from 7 to 49 DIM.
Figure 3. Composition of liver in normal control cows (○) and normal cows treated with glucagon (●) from 21 to 35 DIM. Significant differences between treatments at a given time were indicated by * $P < 0.05$ or ** $P < 0.01$. A) Glycogen: Time × treatment effects between d 20 and d 23: $P < 0.03$; d 20 and d 27, $P < 0.21$; d 20 and d 35, $P < 0.03$; and, d 20 and d 38, $P < 0.003$. B) Triacylglycerols: Time × treatment effects between d 20 and other time: $P > 0.05$. 
(when results were compared on the basis of paired t test). Liver TAG did not differ between NC and NTG cows at any time ($P > 0.05$), and glucagon did not significantly affect liver TAG.

The pattern of change for total liver lipids was similar to that of TAG in both groups (data not shown). Glucagon infusions did not affect total liver lipids. Overall average concentrations of total lipids were approximately 2% greater than those of liver TAG, which would be largely accounted for by structural phospholipids. Liver DM percentage did not change significantly over time in these normal cows; however, it was greater by 3.9% ($P < 0.05$) and 4.6% ($P < 0.03$) in NC cows than in NTG cows at 23 and 28 DIM, respectively.

**Milk Production and Composition**

Average daily milk production of NTG cows was significantly less than that of NC cows at wk 5 postpartum, which was the second week of glucagon infusions (Figure 4). When compared with wk 3, average daily milk production of NTG cows decreased by $2.6 \pm 0.9$ ($P < 0.02$) and $3.0 \pm 0.9$ ($P < 0.009$) kg at wk 4 and wk 5 respectively, whereas milk production of NC cows increased by $2.2 \pm 1.4$ ($P < 0.16$) and $3.7 \pm 2.1$ ($P < 0.02$) kg at those times. During the first week after glucagon infusions ended, milk production in the NTG group increased by $3.3 \pm 0.6$ ($P < 0.0003$) kg, (i.e. wk 6 compared to wk 5). Therefore, daily milk production was decreased only transiently by glucagon infusions.

Milk protein percentages (Figure 5A) had decreased by actual values of $0.47 \pm 0.05\%$ ($P < 0.0001$) at d 2 and by $0.22 \pm 0.07\%$ ($P < 0.01$) at d 13 of glucagon infusions when
Figure 4. Average daily milk production by week in normal control cows (■) and normal cows treated with glucagon (▲) from 21 to 35 DIM. Significant differences between treatments at a given time are indicated by * $P < 0.05$. Time × treatment effects between wk 3 and wk 4, $P < 0.002$; wk 3 and wk 5, $P < 0.0005$; wk 3 and wk 6, $P < 0.05$; and wk 5 and wk 6, $P < 0.05$.

compared with the preinfusion baseline at 20 DIM. Two days after glucagon infusions ended, milk protein percentages had increased by $0.28 \pm 0.07\%$ ($P < 0.003$) when compared with d 13 of glucagon infusions. There were no significant changes in milk protein percentages in NC cows. Because both milk production and milk protein percentage decreased, average daily milk protein yield in NTG cows deceased to 76% and 78% of that in NC cows at d 2 and d 13 of glucagon infusions (data not shown). Obviously, milk protein synthesis was decreased significantly, but only temporarily by glucagon infusions. Although
Figure 5. Percentages of protein, fat, and lactose in milk in normal control cows (o) and normal cows treated with glucagon (●) from 21 to 35 DIM. Significant differences between treatments at a given time are indicated by * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$. A) Milk protein percentage: Time × treatment effects between d 20 and d 23, $P < 0.001$; d 20 and d 34, $P < 0.24$; and, d 34 and d 37, $P < 0.001$. B) Milk fat percentage: Time × treatment effects between d 20 and d 23, or d 34, and d 34 and d 37, $P > 0.05$. C) Milk lactose percentage: Time × treatment effects between d 20 and d 23, $P < 0.001$; d 20 and d 34, and, d 34 and d 37, $P > 0.05$. 


A

Milk protein (%)

Time postpartum (d)

Glucagon

B

Milk fat (%)

Time postpartum (d)

Glucagon

C

Milk lactose (%)

Time postpartum (d)

Glucagon
milk fat percentages were significantly less at 34 and 49 DIM in NTG cows than in NC cows (Figure 5B), glucagon infusions did not affect milk fat percentages because of nonsignificant time \times treatment interactions \((P > 0.05)\). In addition, there were no significant changes in milk fat percentages within both the NTG and NC groups before, during, and after infusions.

Milk lactose percentage increased in actual values by 0.12 ± 0.03% at d 2 of glucagon infusions when compared with the preinfusion baseline of 20 DIM in NTG cows \((P < 0.005)\). Also, milk lactose percentage at d 2 of glucagon infusions (Figure 5C) was greater in NTG cows than in NC cows \((P < 0.007)\). Average daily yield of milk lactose, however, was not significantly affected by glucagon infusions \((P > 0.05, \text{ data not shown})\).

Despite temporal changes in milk production and composition during infusions of glucagon, 305-d mature equivalents of milk, milk fat, and milk protein production per lactation between NTG and NC cows were not affected by glucagon infusions. Neither did 305-d mature equivalents of milk, milk fat, and milk protein production differ between the current and the previous lactation in both the NTG and NC groups (data not shown).

**Feed Intake and BCS**

Average daily feed intakes in NTG cows were significantly less than intakes in NC cows during glucagon infusions (Figure 6), but time \times treatment effects were not significant \((P > 0.05)\). Feed intakes for NC cows increased gradually after calving with a difference of 4.62 ± 1.38 kg between wk 3 and wk 5 \((P < 0.008)\), whereas feed intakes for NTG cows increased only 1.12 ± 1.25 kg from wk 3 to wk 5 \((P < 0.40)\). Feed intakes for NTG cows increased rapidly by 3.06 ± 1.25 kg at wk 6 after glucagon infusion ended when compared
Figure 6. Average daily feed intake by week in normal control cow (●) and normal cows treated with glucagon (□) from 21 to 35 DIM. Significant differences between treatments at a given time are indicated by * $P < 0.05$ or ** $P < 0.01$. Time × treatment effects between wk 3 and wk 4, or wk 5, or wk 6. P > 0.05: wk 5 and wk 6. P < 0.07.

with wk 5 ($P < 0.04$). Thus, glucagon infusions hindered the normal increases of feed intakes with the progression of lactation as seen in NC cows. Although BCS were slightly lower in NTG cows at 14 and 35 DIM. BCS did not differ between NC and NTG groups at any time (data not shown).
DISCUSSION

This report presents results from the first known long-term administration of glucagon to lactating dairy cows. Normal cows were used to provide background information for further studies involving the use of glucagon to alleviate fatty liver in early lactation cows. The metabolic effects of glucagon infusions into early-lactation dairy cows were evaluated by measuring changes of liver components, plasma metabolites and hormones, milk composition and yield, and feed intake before, during, and after glucagon. Intravenous administration of glucagon for 14 d did not show major detrimental effects that would indicate that glucagon could not be used for preventative or therapeutic purposes in high-producing cows. There were some short-term effects related to production performance, however, that are mildly, but not seriously, counterproductive. Such effects include: first, a temporary delay of the increase in feed intake that normally takes place in early lactation, second, a slight decrease in daily milk production, and third, a decrease in the concentration and yield of milk protein during the 2 wk when glucagon was infused.

Most importantly, there were major improvements in both the carbohydrate and lipid status of these high-producing, early-lactation cows. Concentrations of blood glucose were increased significantly and immediately when infusions of glucagon began and remained increased throughout the infusions, indicating that glucose utilization is less than glucose production. The major utilization of glucose in lactating dairy cows is for milk production, which may account for up to 80% of total glucose utilization depending on the level of milk production (14). Glucose is used mainly for synthesis of milk lactose. Because the total daily lactose production was not changed and average daily milk production was decreased...
slightly by glucagon infusions, glucose utilization for milk production may have decreased slightly. Glucose utilization by muscle and adipose tissue, however, may have been increased slightly by increased plasma insulin and glucose during infusions of glucagon. Therefore, overall glucose utilization at least would not decrease during glucagon infusions. Also, glucose that was absorbed from the gastrointestinal tract could not have been increased by glucagon because feed intakes did not increase during glucagon infusions as occurred in NC cows. Thus, increased plasma glucose probably results from increased hepatic glucose production. Because liver glycogen concentrations in NTG cows were increased dramatically during the late part of glucagon infusions, despite initial decreases, hepatic gluconeogenesis most likely was increased by glucagon.

Glucagon is known to increase gluconeogenesis both in vitro and in vivo (10, 11, 13). Glucagon infusions at high concentrations for a few hours increased the conversion of $^{14}$C-alanine to $^{14}$C-glucose in intact dogs, overnight fasted humans (10, 11), and sheep (6). Glucagon stimulates gluconeogenesis by affecting key regulatory enzymes found in hepatocytes and by increasing availability of gluconeogenic substrates in hepatocytes. A glucagon-induced, increased selective uptake of amino acids by the liver has been shown in many studies in nonruminants (4, 11, 20) and in ruminants (6, 9). If the supply of amino acids from extrahepatic tissues is not increased but the hepatic extraction of amino acids is increased, it follows that plasma amino acids should be decreased. A decrease in concentrations of plasma amino acids is a common feature during both hyperglucagonemia and exogenous glucagon infusions. Flakoll et al. (20) found that total plasma amino acids decreased by 17% in dogs when plasma glucagon was increased 4-fold while plasma insulin
was kept relatively unchanged. Moreover, a glucagon deficiency caused by somatostatin increased concentrations of plasma amino acids (5). Plasma amino acids were not measured in the present study, but decreases in both the percentage and yield of milk protein are consistent with decreased concentrations of plasma amino acids. This relationship occurs because milk protein synthesis is controlled by availability of plasma amino acids, and decreases in total plasma amino acids or a specific amino acid deficiency would severely impede milk protein synthesis. On the other hand, propionate is a major substrate for gluconeogenesis, but the effect of glucagon on utilization of propionate for gluconeogenesis is still uncertain. Glucagon stimulated hepatic uptake of propionate and gluconeogenesis in perfused sheep liver (25), but it did not affect in vivo utilization of propionate for glucose synthesis in sheep (8). It is unclear whether gluconeogenesis from propionate was increased by glucagon infusion in our current study.

Liver glycogen decreased rapidly during the first 2 d after glucagon infusions began, which results from glucagon-stimulated net glycogenolysis. The repletion of liver glycogen by d 7 of glucagon infusions and its continued increase in the late part of infusions and even 3 d after infusions ended indicate a potent increase in glycogen synthesis in the liver of NTG cows. Glucagon stimulates glycogen phosphorylase very potently and induces glycogenolysis (30). However, the effect of glucagon on glycogen waned within 3 h of glucagon administration, even when counterregulatory insulin secretion did not occur in dogs fasted overnight (10). The reason for increases in glycogen after its initial depletion in our study is uncertain. In the first few hours after glucagon infusions begun, increased plasma insulin probably counteracted glucagon to prevent further net glucagon breakdown.
However, liver glycogen increased, while plasma insulin did not increase during the second week of glucagon infusions in NTG cows. In addition, in another study with ketosis-susceptible cows, the pattern of changes in liver glycogen was very similar to that of normal cows, but plasma insulin did not increase except for the first few hours during 14-d infusions of glucagon (unpublished data). Thus, factors other than insulin could regulate glycogen synthesis. It was hypothesized by Radziuk et al. (27) that metabolites, i.e., gluconeogenic substrates, are the primary regulatory factors in glycogen synthesis. Although the mechanisms for this regulation are unidentified, they found that lactate stimulated glycogen synthesis during glucose loading (27). Because glucokinase activity is very low in ruminant liver (2), glycogen probably is synthesized primarily from gluconeogenic substrates. Our results suggest that this process could be stimulated by increased gluconeogenesis during glucagon infusion.

The normal increase in feed intake usually seen in early lactation cows was decreased in magnitude by infusions of glucagon into NTG cows. Injections of glucagon decreased feed intake in rats, dogs, chickens, rabbits, and humans (21), and in sheep (15). Considering the major increases in plasma glucagon concentrations and the small inhibition of feed intake caused by glucagon infusions, it is likely that glucagon at physiological levels is not important in regulating feed intake. The reason for the decrease in daily milk production in NTG cows during glucagon infusions is unclear. The decrease may relate to feed intakes being limited by glucagon infusions in those cows. Milk production in dairy cows is affected by many factors, among which substrate supply and mammary blood flow are primary. Whether glucagon can directly affect the mammary gland is unclear because there is no
evidence of glucagon receptors in the mammary gland (22). The limitation in milk production is transient because milk production per lactation in NTG cows did not differ from that of NC cows or from the previous lactation.

The most important observation with respect to lipids is that infusions of glucagon at 10 mg/d did not give any indication of increasing lipolysis from adipose tissue and thereby of increasing concentrations of NEFA in blood. This is not the response expected based upon textbooks of physiology and biochemistry, which consistently indicated that glucagon is lipolytic with respect to adipose tissue. Average concentrations of NEFA were generally less in NTG than in NC cows, but these differences were present even before glucagon was infused. Plasma NEFA concentrations decreased during glucagon infusions in sheep (3) and in mid-lactation cows (unpublished data), which may be explained by increased concentrations of plasma insulin during glucagon infusions and by strong inhibitory effects of insulin on lipolysis. Thus, it is still unclear whether glucagon has any direct lipolytic effect in ruminants because concentrations of plasma insulin increased during glucagon infusions. Further studies are needed to clarify the effect of glucagon on lipolysis in ruminants. Concentrations of TAG in the liver decreased in NC and NTG cows with progression of lactation. Unfortunately, TAG percentages in livers of NTG cows were decreased even before infusions of glucagon began, which prevented a decisive conclusion about whether glucagon causes an increased rate of removal of TAG from livers of normal dairy cows. Further studies are needed on this possible effect of glucagon, also.
CONCLUSIONS

The main metabolic and performances changes caused by 14-d infusions of glucagon at a pharmacological dose in normal early-lactation dairy cows in this study are: a) an initial net hepatic glycogenolysis followed by an increase in net glycogen synthesis, b) constant increases in plasma glucose during glucagon infusions, c) decreases in production of milk and milk protein, but no changes in milk lactose yield during glucagon infusions, d) a small inhibition of voluntary feed intake during glucagon infusions, and e) increases in plasma insulin during the first week of glucagon infusions. Assuming that glucose utilization did not decrease during glucagon infusions, based on milk data and possible increases of glucose oxidization in muscles and adipose tissue by increased plasma insulin and glucose, the results suggest that glucagon in excess increases glucose production by increasing net glycogenolysis initially and then by promoting gluconeogenesis partly from an increased hepatic uptake of plasma amino acids. The results of this study are not indication of a net lipolytic effect from glucagon infusions in dairy cows.

ACKNOWLEDGMENTS

The authors acknowledge R. Lenius of Swiss Valley Farms Co. Davenport, IA for analyzing milk samples. Appreciation is extended to J. Kent for intensive care of cows, to D. Crawley and C. Achen for provision of cows, to S. Deng for analyzing liver samples, and to Dr. D. F. Cox and G. Bobe for assistance in statistical analyses.
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REGULATION OF mRNA EXPRESSION FOR GLUCONEOGENIC ENZYMES
DURING GLUCAGON INFUSIONS INTO LACTATING COWS

A paper prepared for submission to the Journal of Dairy Science
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ABSTRACT

Effects of glucagon infusions on mRNA expression for gluconeogenic enzymes in lactating cows were studied. Normal and ketosis-susceptible cows were assigned into either glucagon-treated or control groups to give a 2 x 2 factorial design. Glucagon infusions at 10 mg/d began at d 21 postpartum for 14 d. In normal cows, glucagon infusions increased plasma glucagon about 6-fold and increased plasma glucose 19%. Phosphoenolpyruvate carboxylase (PEPCK) mRNA decreased during wk 1 of glucagon infusions while plasma insulin increased. The susceptible cows had low plasma glucose and high BHBA before infusions. Glucagon infusions increased plasma glucagon about 8-fold and glucose 43%, but did not significantly change plasma insulin and hepatic PEPCK mRNA. PEPCK mRNA was greater overall in susceptible cows than in normal controls. In another experiment with mid-lactation cows, 3.5-h glucagon infusions at 15 mg/d rapidly increased plasma glucose, insulin and decreased plasma BHBA and hepatic glycogen. PEPCK mRNA decreased 41%, but mRNA for pyruvate carboxylase increased 50% by the end of 3.5-h glucagon infusions, and mRNA for fructose 1, 6 bisphosphatase was not influenced. We conclude that PEPCK gene expression in normal cows is inhibited potently by insulin to balance elevated carbohydrate
status during glucagon infusions. PEPCK mRNA expression probably is not involved in the pathogenesis of bovine ketosis.

INTRODUCTION

Concentrations of plasma glucose are maintained within narrow ranges in both ruminants and nonruminants to maintain normal body functions. In nonruminants, the liver regulates glucose homeostasis in many physiological and nutritional conditions through key regulatory enzymes. These regulatory enzymes of glycogen metabolism, gluconeogenesis, and glycolysis are controlled acutely and chronically by hormones and nutrients. Metabolic fluxes of substrates through individual metabolic pathways are enhanced or inhibited accordingly (37).

In dairy cows, ketosis is a common metabolic disorder associated with disturbances of both carbohydrate and lipid metabolism. Ketosis is characterized by hypoglycemia, depletion of liver glycogen, ketonemia, and fatty liver. The pathogenesis of ketosis is not known clearly; however, it is generally thought to result from inadequate availability of endogenous glucose (2). It is still questionable whether the gluconeogenic capacity of the liver of ketosis-susceptible cows is impaired, thereby causing ketosis (2. 35). An experimental model of ketogenesis in lactating dairy cows has been developed. This model, which was identified as feed restriction and butanediol feeding (FRBD), has been used to induces a metabolic state that resembles ketosis (34).

Glucose metabolism in ruminants differs from that of nonruminants. First, because most dietary carbohydrate is fermented in the rumen and only small amounts of glucose are
absorbed directly from the gastrointestinal tract, over 90% of the glucose supply of ruminants depends on a state of continuous gluconeogenesis, which increases after feeding (29, 31). Second, demands for glucose in lactating ruminants are great because a large proportion of available glucose is used for lactose synthesis during the whole period of milk production.

Third, ruminants oxidize limited amounts of glucose and use acetate rather than glucose for fatty acid synthesis (4). As a result, plasma glucose concentrations are much lower in ruminants than in nonruminants.

In general, gluconeogenic rate is controlled by substrate availability, quantities of rate-limiting enzymes, and the state of activation of rate-limiting enzymes, all of which are influenced by hormones. The availability of gluconeogenic substrates is a major determinant for the rate of gluconeogenesis in ruminants (31). The mechanisms of hormonal control of gluconeogenesis and its enzymes have not been studied intensely in ruminants; however, hormones such as glucagon, insulin, growth hormone, glucocorticoids, and catecholamines play important roles in regulating glucose metabolism (6, 33). Comparative studies in ruminants and nonruminants of lactation, ketosis, fasting, or hormonal treatments have shown that gluconeogenic enzyme activities from ruminant liver were sometimes inconsistent and differed from those in nonruminants (3, 4, 5, 19). For example, PEPCK (EC 4.1.1.32) adapted little to changes in physiological or nutritional conditions (4, 47).

Regulatory differences may exist among species because nutrition and endocrine status varies widely among species. Metabolic regulation at the molecular level can be understood by using new molecular technology to study physiological conditions in animals and cells (21).

In nonruminants, extensive studies have been made of nutritional and hormonal regulation of
gene expression for some enzymes of glucose metabolism both in vivo and in vitro (25, 37), but very few studies of this kind have been done with ruminants.

Ruminants are highly dependent on gluconeogenic pathways for regulation of blood glucose. Glucose-6-phosphatase (EC 3.1.3.9), fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), pyruvate carboxylase (EC 6.4.1.1), and PEPCK are the four key regulatory enzymes of gluconeogenesis. We hypothesize that hormonal regulation of gene expression for these gluconeogenic enzymes is critical in controlling glucose homeostasis in response to hormonal status, as well as in development of ketosis in dairy cows. The first goal of this study was to determine whether several metabolic variables and gene expression of selected gluconeogenic enzymes were influenced by infusions of glucagon into normal lactating dairy cows. Also, we induced ketosis by using the FRBD protocol to approach the second goal, which was to determine whether expression of the PEPCK gene is involved in the pathogenesis of ketosis and the response of ketosis-susceptible dairy cows to glucagon infusions. Accomplishing these two goals will contribute to greater understanding of carbohydrate regulation in normal cows and to understanding the molecular pathogenesis of ketosis.

MATERIALS AND METHODS

Long-term Infusions of Glucagon into Dairy Cows

The objective was to determine chronic changes of PEPCK mRNA and its metabolic relationships by modifying hormonal and nutritional conditions in dairy cows. Twenty normal multiparous Holstein cows with an average body condition score of approximately
3.6. and 20 obese multiparous Holstein cows with body condition scores over 4 on a scale of 1 to 5 at d 15 prepartum were used. Normal cows and obese cows (cows that were susceptible to ketosis) were selected on the basis of having ratios of liver triacylglycerols to glycogen (wt/wt) of less than 1.5 and greater than 2.5 at 6 DIM, respectively. Four (two normal and two susceptible) cows calving in the same 2-wk interval were considered a block. The two normal cows were assigned randomly to two groups identified as normal controls (NC) or normal cows treated with glucagon (NTG). The two susceptible cows were assigned randomly as susceptible controls (SC) or susceptible cows treated with glucagon (STG). Thus, there were 10 blocks or replications of a 2 x 2 factorial design with two nutritional backgrounds (normal and obese) and two levels of glucagon (0 and 10 mg/d).

Normal cows were fed a TMR and grass hay according to NRC recommendations during the prepartum period. Susceptible cows were made obese by feeding extra corn at 5.5 kg/d during the last 30 d prepartum. The general layout of the experiment is shown in Figure 1. Both groups of susceptible cows received FRBD from 14 to 42 DIM to induce ketosis. During FRBD, the susceptible cows were feed-restricted to about 80% of the maximum intake determined during the two consecutive days just before FRBD. Also, a ketone body precursor, 1,3-butanediol, was added to the diet. Except for FRBD, treatment and management of susceptible cows after calving was the same as for normal cows. From 21 to 35 DIM, both NTG and STG cows were infused continuously through one jugular vein with glucagon (10 mg/d), while both NC and SC cows were infused with 0.15 M NaCl (20 ml/h). Nine liver biopsies (days identified in Figure 1) and daily blood samples were obtained during the 42 d experimental period from 7 to 49 DIM. Details of catheterization, infusion
Figure 1. Experimental protocol for long-term infusions of glucagon. Four groups of cows as described in “Materials and Methods” were used in the experiment began from 7 to 49 DIM. Only the susceptible cows received feed restriction and butanediol (FRBD) from 14 to 42 DIM. Glucagon was infused intravenously into each group of normal and susceptible cows from 21 to 35 DIM. LB stand for liver biopsy.

conditions, liver biopsy and other sampling procedures, and feeding were described elsewhere (43).

Short-term Infusions of Glucagon into Dairy Cows

The objective was to determine acute effects of glucagon infusions into normal dairy cows on the regulation and time course of PEPCK mRNA and other metabolic parameters. Two days before infusions, polyethylene catheters were implanted into each jugular vein. Five mid-lactation Holstein cows were infused continuously via one catheter with glucagon for 3.5 h at 15 ng/(kg×min). Four weeks later, they were infused with 0.15 M NaCl (20 ml/h) to give control values. The dosage of glucagon (15 mg/d) was 1.5 fold greater than for the
long-term study. Five liver biopsies were obtained for each infusion (at 0, 0.75, and 3.5 h during infusions, and at 2 h and 3.5 h postinfusion). Each liver sample weighed about one gram. A series of blood samples was collected pre, during, and postinfusion from the other catheter. Sampling was begun at about 8 a.m., just after feeding and milking, and ended at about 2 p.m. Feed and water were available to the cows during the experimental period.

RNA Isolation and Northern Blotting

Frozen liver samples stored at -80 °C were powdered by grinding in liquid N. and total RNA was extracted from about 100 mg of tissue by using TRizol™ Reagent (Life Technologies, Inc., Grand Island, NY). Total RNA was quantified spectrophotometrically at 260 nm and stored at -80 °C. Ratios of absorbencies at 260 nm to 280 nm were calculated to determine the quality of RNA isolated. RNA isolation and analyses were done as soon as possible to avoid degradation of mRNA.

Twenty μg of total RNA was electrophoresed through 1% agarose gels containing formaldehyde. The gels were stained with ethidium bromide and, upon exposure to ultraviolet light, the 18S and 28S rRNA could be seen intact and were of similar size across samples. Nucleic acids were transferred to nylon membranes (Magnacharge, MSI, Westboro, MA), and membranes were prehybridized, hybridized, washed, and exposed to x-ray films for 1 to 5 days according to the intensity of radioactivity (40). An about 2 kb cDNA clone for human PC gene encoding the 3’ end (provided by B. H. Robinson, Hospital for Sick Children, Toronto, Ontario) was used first. Then, the membrane was stripped and hybridized with FBPase probe made from a pig cDNA clone of about 1 kb in length, which was
subcloned into Pet-11a with BamHI and Xba (provided by H. J. Fromm, Iowa State University, Ames, IA). Finally, they were hybridized with a probe made from a 2.1 kb insert from the Hind III site of the chicken β-actin cDNA. Probes for these cDNA clones were made by the random primer method using [α-32P]-dCTP (300 Ci/mmol) (RadPrime DNA labeling System, Life Technologies, Inc., Grand Island, NY). The densities of bands were determined by image densitometric analysis at the Iowa State University Image Analysis Facility. Concentrations of β-actin mRNA were used to correct for loaded amounts of total RNA samples, and relative concentrations of PC mRNA and FBPase mRNA were calculated.

Solution Hybridization

Two subclones for rat PEPCK cDNA, PCK.L11 and PCK.L15 were derived from a full-length PEPCK cDNA clone, PCK10 (provided by Dr. R. W. Hanson, Case Western University, Cleveland, OH). The inserts were subcloned in pBluescript KS+, and their sequences were verified so that they could be used as templates for making riboprobe and single strand DNA (ssDNA) for standards in solution hybridization, respectively. An antisense RNA riboprobe containing 417 bp of the PEPCK gene was synthesized by using T3 RNA polymerase and an in vitro transcription kit (MAXIscript™. Ambion, Austin, TX). A 3.4 kb DNA fragment from digestion of PCK.L11 with Sam I was used as a template. The antisense RNA was labeled with [35S]-UTP (10 mCi/ml, specific activity > 1.000), and incorporation rates of radioactive UTP were greater than 50%. ssDNA, which was the sense strand of PEPCK gene, was synthesized by culture of bacteria containing PCK.L15 and co-infection with M13 helper phage.
A range of amounts of ssDNA and 5 μg of total RNA for each sample were hybridized overnight with the riboprobes in solution at 72 °C. Double strand hybrids protected from RNase A and RNase T1 were precipitated by trichloroacetic acid and captured by filtration under vacuum on Whatman GF/C glass filters. Radioactivity was counted by liquid scintillation spectroscopy, and the content of mRNA was calculated by using standard curves. The intra-assay and inter-assay CV were 2.8% and 9.8%, respectively, and the average r² for the standard curves in a total of 24 assays was 0.997 (data not shown).

Analytical Procedures

The methods for analyzing liver glycogen and triacylglycerols, plasma metabolites (glucose, NEFA, and BHBA), and plasma hormones (glucagon and insulin) were described earlier (43). Plasma metabolites for the study of short-term glucagon infusions were determined manually. Plasma glucose was measured by the glucose oxidase method (Glucose Trinder, Sigma Diagnostic, St. Louis, MO). Plasma NEFA were analyzed by a commercial kit (NEFA-C, Wako Pure Chemical Industries, Ltd. Osaka, Japan). Plasma BHBA was measured according to Williamson, et al. (46).

Statistical Analysis

Data from both the long-term and the short-term studies of glucagon infusions were subjected to ANOVA by using the general linear models procedures of SAS. A separate analysis was done for each time period or sampling time point to determine whether mean values for two individual groups differed. The changes over time within each treatment
group were assessed by a paired $t$ test (44) by testing whether differences between two selected times differed from zero. For the short-term study, additional analyses of time $\times$ treatment effects were done on functions of difference between the repeated measures on the same cow to test two differences for glucagon-treated and control cows. Associations between PEPCK mRNA and other metabolic parameters in the long-term study were calculated by Pearson correlation analysis.

**RESULTS**

**Long-term Infusions of Glucagon into Dairy Cows**

Plasma glucagon concentrations in NC cows increased from wk 2 to wk 7 ($P < 0.02$), and the average concentration was greater in NC cows than in SC cows ($P < 0.06$, Table 1). Plasma glucagon concentrations increased 6- to 7-fold in NTG cows and 8- to 9-fold in STG cows during glucagon infusions (Table 1). Plasma insulin concentrations of NC and SC cows increased from wk 2 to wk 7, but the average concentration was greater in NC cows than in SC cows ($P < 0.0007$, Table 1). Concentrations of plasma insulin were significantly greater during wk 1 of glucagon infusions in NTG cows than in NC cows ($P < 0.05$), but did not differ during wk 2 of infusions ($P > 0.05$). Plasma insulin concentrations did not differ between STG and SC cows at wk 1 and wk 2 of infusions ($P < 0.02$), however, plasma insulin in STG cows increased during the first 4 h of glucagon infusions (data not shown).

Plasma glucose concentrations (Table 1) increased slightly from wk 2 to wk 7 in NC cows ($P < 0.05$). Glucose concentrations increased 19% (10.1 mg/ml) over preinfusion baseline during glucagon infusions in NTG cows ($P < 0.0004$) and 43% (17.3 mg/ml) in
Table 1. Weekly average concentrations of plasma glucagon, insulin, glucose and β-hydroxybutyrate (BHBA) in normal controls (NC), normal cows treated with glucagon (NTG), susceptible controls (SC), and susceptible cows treated with glucagon (STG).

<table>
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<th>Postinfusion</th>
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¹ All four groups of cows were infused during week 3 and 4 postpartum either with 0.15 M NaCl (NC and SC) or glucagon (10 mg/d, NTG and STG). The SC and STG groups were subjected to the feed restriction and butanediol protocol from week 3 through week 6.

² The general SEM of that group. Selective statistical conclusions are described in “Results”.

STG cows (P < 0.0001). Plasma glucose decreased at wk 3 compared with wk 2 in SC (P < 0.04) and STG (P < 0.002) cows, after FRBD feeding was begun. FRBD feeding to SC and STG cows ended at wk 6, and in wk 7 glucose concentrations increased in SC (P < 0.01) and STG (P < 0.01) cows (Table 1). Plasma BHBA concentrations (Table 1) were greater both in SC and STG cows before FRBD feeding was initiated (P < 0.006), and BHBA increased
during the FRBD feeding in SC and STG cows ($P < 0.01$). FRBD feeding has been shown previously to increase plasma ketone concentrations (Mills, 1986), and BHBA concentrations at 1 wk after FRBD decreased rapidly ($P < 0.001$).

Concentrations of hepatic PEPCK mRNA (Figure 2) were similar for cows in all treatments at day 7 postpartum ($P > 0.05$). PEPCK mRNA was greater in SC cows that had undergone the FRBD dietary treatment beginning at d 14 postpartum, than in NC cows at d 20 ($P < 0.04$), d 38 ($P < 0.01$), and d 42 ($P < 0.03$) postpartum. PEPCK mRNA concentrations in NTG cows decreased during the first 7 d of glucagon infusions ($P < 0.03$).

![Figure 2](image_url)

**Figure 2.** Concentrations of PEPCK mRNA in normal controls (NC, ○), normal cows treated with glucagon (NTG, ★), susceptible controls (SC, □), and susceptible cows treated with glucagon (STG, ▲). The general SEM are 4.8 for NC cows, 3.7 for NTG cows, 7.6 for SC cows, and 5.3 for STG cows. Treatments of cows were described in "Materials and Methods"; and the statistical analysis was described in "Results".
but increased between d 7 and d 14 of glucagon infusions to near preinfusion levels (Figure 1) at the same time that plasma insulin concentrations were decreasing (Table 1). In SC and STG cows, PEPCK mRNA concentrations seemed to decline during infusions, but did not differ significantly between groups during the entire experimental period ($P > 0.05$).

**Short-term Infusions of Glucagon into Normal Dairy Cows**

During the long-term infusions of glucagon, plasma glucagon and glucose were increased, whereas plasma insulin increased only during the first week of infusions into NTG cows (Table 1). Expression of the PEPCK gene, which was expected to increase during glucagon infusions on the basis of known responsiveness of the PEPCK gene to glucagon, instead declined during infusions (Figure 2). Therefore, short-term infusions of glucagon were conducted with frequent sampling of liver and blood to determine whether PEPCK gene expression was acutely responsive to glucagon.

Plasma glucagon and insulin concentrations increased 8-fold (Figure 3A) and 3.8-fold (Figure 3B), respectively, during the first 2 h of glucagon infusions. Glucagon concentrations remained high throughout infusions, whereas insulin concentrations declined steadily beginning at 2 h of infusions and were still greater than baseline at the end of infusions ($P < 0.003$, Figure 3B). At the completion of glucagon infusions, plasma glucagon decreased rapidly to baseline concentrations, and plasma insulin decreased to below baseline concentrations ($P < 0.03$, Figure 3B).

Liver glycogen concentrations decreased from 3.5% to 2.1% during the first 0.75 h of glucagon infusions (Figure 4A, time × treatment interaction $P < 0.02$) and decreased further
Figure 3. Concentrations of plasma glucagon and insulin in control (○) and glucagon-treated (●) cows. A) Glucagon: Time × treatment effects between pre-infusion and during infusion, $P < 0.001$; between pre-infusion and post-infusion, $P < 0.28$. B) Insulin: Time×treatment effects between pre-infusion and during infusion, $P < 0.005$; between pre-infusion and post-infusion, $P < 0.03$. 
Figure 4. Concentrations of liver glycogen and plasma metabolites in control (○) and glucagon-treated (●) cows. A) Liver glycogen: Time × treatment effects between 0 and 0.75 h, $P < 0.02$; between 0 and 3.5 h, $P < 0.02$; between 0 and 5.5 h, $P < 0.01$; between 0 h and 7 h, $P < 0.03$. B) Plasma glucose: Time × treatment effects between pre-infusion and during infusion, $P < 0.001$; between pre-infusion and post-infusion, $P < 0.89$. C) Plasma nonesterified fatty acids: Time × treatment effects between pre-infusion and during infusion, $P < 0.31$; between pre-infusion and post-infusion, $P < 0.25$. D) Plasma β-hydroxybutyrate: Time × treatment effects between pre-infusion and during infusion, $P < 0.36$; between pre-infusion and post-infusion, $P < 0.89$. 
through the remainder of infusions. Liver glycogen of glucagon-treated cows did not increase during the 3.5 h postinfusion period. Plasma glucose concentrations increased 1.5-fold during the first hour of glucagon infusions (Figure 4B). At 2 h of glucagon infusions, insulin was still at its maximal concentration (Figure 3B), but plasma glucose concentration had declined from peak values (Figure 4B, $P < 0.04$). Plasma glucose concentrations were similar in preinfusion and postinfusion periods ($P > 0.05$). Plasma NEFA concentrations decreased by 115 μeq/L during infusions ($P < 0.03$) and increased after infusions ended (Figure 4C). Plasma BHBA was not influenced by short-term infusions of glucagon (Figure 4D).

PEPCK (data not shown) and PC mRNA (Figure 5) were detected as single mRNA transcripts by Northern blotting, and corresponded to 2.8 kb and 4.2 kb, respectively, which agrees with rat mRNA sizes (14, 27). FBPase, however, was detected as a major transcript of 1.4 kb that agrees with rat FBPase mRNA size (18) and a minor transcript of 2.1 kb, and both transcripts were integrated by scanning densitometry.

Concentrations of hepatic PEPCK mRNA as determined by solution hybridization tended to increase during the first 0.75 h of glucagon infusions ($P < 0.1$), but PEPCK mRNA concentrations at 3.5 h of glucagon infusions decreased to 59% of preinfusion baseline (Figure 6A, $P < 0.03$) and remained decreased through 2 h postinfusion. Interestingly, PEPCK mRNA concentrations at 3.5 h of infusions increased 31% in control cows that were infused with 0.15M NaCl ($P < 0.01$) and remained increased at 2 h postinfusion.

The abundance of PC mRNA as determined by Northern blotting and scanning densitometry increased 1.5-fold at the end of infusions ($P < 0.006$) and remained increased
Figure 5. Northern blotting of pyruvate carboxylase (PC) mRNA and fructose 1,6 bisphosphatase (FBPase) mRNA in dairy cows in response to glucagon infusions. Treatments of cows were described in “Materials and Methods”. Lanes 1, 2 and 3 are 0, 0.75 and 3.5 h of glucagon infusions, respectively. Lanes 4 and 5 are 2.0 and 3.5 h post-infusion, respectively.
Figure 6. Concentrations of mRNA for liver gluconeogenic enzymes in control (□) and glucagon-treated (■) cows. A) Phosphoenolpyruvate carboxylase (PEPCK) mRNA: Time x treatment effects between 0 and 0.75 h, $P < 0.20$; between 0 and 3.5 h, $P < 0.01$; between 0 and 5.5 h, $P < 0.005$; between 0 and 7 h, $P < 0.10$. B) Pyruvate carboxylase (PC) mRNA: Time x treatment effects between 0 and 3.5 h: $P < 0.21$; between 0 and 5.5 h, $P < 0.08$; between 0 and 7 h, $P < 0.27$. C) Fructose 1, 6 bisphosphatase (FBPase) mRNA: Time x treatment effects between 0 and 3.5 h, $P < 0.26$; between 0 and 5.5 h, $P < 0.65$; between 0 and 7 h, $P < 0.98$. 
even at 3.5 h postinfusion (Figure 6B). However, the time × treatment effects were not significant, which was probably due to high variability. FBPase mRNA was unaffected by glucagon infusions (Figure 6C).

**DISCUSSION**

**Regulation of mRNA for PEPCK, PC, and FBPase by Glucagon Infusions**

By performing multiple liver biopsies during glucagon infusions, we were able to study the temporal regulation of gene expression of some gluconeogenic enzymes in dairy cows. Infusions of glucagon in the short-term and long-term experiments caused a net decrease in hepatic expression of PEPCK mRNA. These decreases were especially apparent in the short-term study where abundance of PEPCK mRNA had decreased after 3.5 h of glucagon infusions. The decrease in PEPCK mRNA expression by glucagon infusion is contrary to the known activation of PEPCK transcription by glucagon or cAMP (8, 14, 15, 30), but is explained by the stimulation of endogenous insulin secretion during glucagon infusions. Insulin is a potent down-regulator of PEPCK transcription in vitro and in animals (14, 20, 23, 28, 41). When plasma insulin concentrations were greater, such as in the NTG cows during wk 1 of glucagon infusions, PEPCK mRNA declined and reached a nadir at d 35 postpartum, near the time of maximal plasma insulin (43). During wk 2 of glucagon infusions, PEPCK mRNA increased while plasma insulin declined. Moreover, in SC and STG cows that were made susceptible to ketosis by accumulation of fat in the liver, plasma insulin concentrations increased little in response to glucagon infusions and PEPCK mRNA did not decrease as much as it decreased in normal cows. In the SC cows, plasma
concentrations of insulin and glucose were lower than those for any other treatment groups, and abundance of PEPCK mRNA was the greatest nearly throughout the experiment. On the basis of the inverse relationship between PEPCK mRNA and plasma insulin concentrations, we conclude that PEPCK mRNA expression was down-regulated by endogenous insulin during glucagon infusions.

Many regulators affect PEPCK gene transcription, and effects of cAMP and insulin have been defined most clearly (25). In H4IIE hepatoma cells, Granner et al. (23, 42) found that cAMP increased PEPCK gene transcription within 5 min. transcription was maximal within 30 min. and insulin at 5 nM decreased PEPCK transcription by 50% within 15 min. Insulin repression of PEPCK occurred both in untreated cells and in cells treated first with 8-(4-chlorophosphorylthio)-cAMP, dexamethasone, or both. From this study and others, insulin is understood to play a dominant inhibitory role in the transcriptional regulation of PEPCK (22, 23, 32, 41, 42). Plasma glucagon increased much greater than did insulin in the current study, but the dominant inhibitory role of insulin in vivo is nevertheless sufficient to override glucagon stimulation and suppress PEPCK transcription, in agreement with previous studies (22, 23, 42).

The PC transcript was expressed as a single transcript of 4.2 kb, which is similar to that of non-ruminant species (27). PC increased during the first three hours of glucagon infusions, but molecular regulation of the PC gene is not as well understood as that for PEPCK. Because PC is a key gluconeogenic enzyme, we expected its transcriptional regulation to be similar to that of PEPCK. Like PEPCK, the amount of PC protein was increased by fasting and diabetes (39). The pattern of gene expression in the current study
may suggest glucagon or cAMP stimulation of PC mRNA expression with little repression by insulin, but further studies on hormonal regulation of PC gene are warranted.

FBPase was detected as two transcripts, both of which were varied together. FBPase transcription in rats is activated by cAMP but, similar to PEPCK, is repressed by insulin (17, 18). In the current study, FBPase mRNA abundance was not affected by glucagon infusions. FBPase mRNA has a half-life of 6 h (18), whereas the infusion period lasted 3.5 h. The relatively long half-life of FBPase mRNA can explain the lack of FBPase repression in the current study, whereas abundance of PEPCK mRNA could be more sensitive to transcriptional down-regulation because the half-life of its mRNA is approximately 0.5 h (36). Alternatively, PC gene regulation could have a differently sensitivity to insulin and glucagon than does PEPCK.

**Effects of Glucagon Infusions on Glucose Metabolism in Normal Cows**

For the short-term glucagon infusions, cows were infused with 1.5-fold the dose of glucagon that was used in the long-term study. As a result, net increases in concentrations of plasma glucose and insulin were greater than those observed in the long-term study. Liver glycogen decreased 2.1% (actual value) over 3.5 h of glucagon infusions, but two-thirds of this decrease occurred within the first 0.75 h of glucagon infusions (Figure 4). This result confirms the initial glycogenolysis that was observed in our study of long-term glucagon infusions (43). By using isotopic techniques, Cherington et al. (12) also found that initial glycogenolysis in overnight fasted dogs occurred within 3 h of glucagon infusions, but waned with time even when increases in plasma insulin were prevented. Decreases in
concentrations of PEPCK mRNA by the end 3.5-h glucagon infusions may reflect a negative response to increases in plasma glucose concentrations caused by initial net glycogenolysis during glucagon infusions.

Although gluconeogenic rates and hepatic glucose output were not determined directly by isotopic techniques in our studies, increased gluconeogenesis caused by long-term infusions of glucagon into normal cows was suggested indirectly (43). Liver glycogen decreased at d 2 of glucagon infusions and but was restored completely by d 7 of glucagon infusions. Plasma insulin increased during wk 1 of glucagon infusions and could have stimulated glucose oxidation in the peripheral tissues. Therefore, constant increases in plasma glucose must result from initial glycogenolysis and later gluconeogenesis during wk 1 of glucagon infusions. Glucagon can stimulate gluconeogenesis by several means. First, glucagon increased hepatic uptake of plasma amino acids in dogs, humans (13), and sheep (10). Second, glucagon increased fatty acid oxidation in perfused liver and in isolated hepatocytes (16), thereby increasing cellular concentrations of acetyl-CoA to activate PC. Enzymatic activity of PC was increased by glucagon infusions in sheep (11); and our data also show that PC mRNA was increased by glucagon infusions. Actually, pyruvate carboxylation by isolated hepatocytes was increased by glucagon (1, 16, 38). All these factors can increase the availability of OAA, the substrate for PEPCK, in hepatocytes, which would increase the flux rate through PEPCK (25) and promote gluconeogenesis. Third, glucagon caused increased mitochondrial matrix volume, activated respiratory chain, and increasing pyruvate carboxylation, citrulline synthesis, and glutaminase activity in isolated
hepatocytes (24, 38). Stimulation of these mitochondrial processes could promote
gluconeogenesis.

In overnight fasted dogs, simultaneous 4-fold increases of glucagon and insulin for 3 h caused significantly decreased glucose production and increased glucose utilization, and insulin was thought to be a potent inhibitor of the stimulatory effects of glucagon on
glycogenolysis and gluconeogenesis (45). In sheep, insulin at physiological ranges inhibited
gluconeogenesis (9). Our data suggest that PEPCK mRNA expression is inhibited in vivo by insulin, even in the presence of high glucagon concentrations. PEPCK has no known
allosteric effectors and is not regulated by covalent modification, and the main regulation was
at the mRNA level (25). Thus, decreases in PEPCK mRNA may reflect sensitive hormonal
regulation of the PEPCK gene to balance increases in net glycogenolysis and
gluconeogenesis during glucagon infusions.

Bassett (7) discussed the balance between the effects of insulin and counter-regulatory
hormones or substrate supply in the early postprandial period when gluconeogenesis and
hepatic glucose output are highest. After feeding, substrate supply and probably counter-
regulatory hormones are increased, but insulin also is stimulated markedly by feeding.
therefore, insulin would act to minimize the conversion of amino acids and propionate to
-glucose (7). Thus, it would be interesting to study regulation of PEPCK gene expression by
dietary factors such as feeding-fasting-refeeding and alterations of diet composition in
ruminants.
Metabolic Variations of Ketosis-susceptible Cows

Our ketosis model, FRBD, consisted of overfeeding during the dry period to make cows obese and FRBD feeding in the early lactation period. These obese cows had 15.3% hepatic lipids and decreased hepatic glycogen content at d 7 postpartum and were susceptible to ketosis (26). They became hyperketonemic, and some later developed clinical ketosis by FRBD feeding during the experiment (26).

The pattern of PEPCK mRNA in these cows differed somewhat from that of normal cows. Plasma insulin concentrations were significantly less in susceptible cows than in normal cows at d 7 postpartum, but PEPCK mRNA abundance was similar in all groups (Figure 2). This result suggests that the susceptible cows may have failed to up-regulate PEPCK mRNA expression normally in the immediate postpartal period. Later, during glucagon infusions, plasma insulin concentrations did not increase in STG cows, but PEPCK mRNA abundance did not increase as expected by stimulation of glucagon on PEPCK transcription. PEPCK gene expression in susceptible cows may be complicated by fatty liver, FRBD feeding, and glucagon infusions, and it is very difficult to define factors that have affected PEPCK mRNA abundance in these cows. However, it is clear that mRNA abundance in SC cows was greater overall than that in NC cows.

Plasma glucose concentrations were significantly less in susceptible cows than in normal cows before glucagon infusions, but the net increase in average concentration of plasma glucose was greater in glucagon-treated cows than in NTG cows during glucagon infusions (17.3 vs. 10.1 mg/dl, Table 1). Also, plasma concentrations were affected significantly by FRBD feeding. Average concentration of plasma glucose during the first
week of FRBD feeding decreased compared with that one week before FRBD feeding in susceptible cows. In contrast, average concentration of plasma glucose in one week after FRBD was greater than during the last week before FRBD ended. The responses in plasma glucose of susceptible cows to glucagon infusions and to FRBD feeding are consistent with the observations that substrate supply greatly regulates gluconeogenesis in ruminants (31) and that PEPCK mRNA expression and enzyme activity (5) did not decrease in cows with fatty liver and hyperketonemia.

**CONCLUSIONS**

The sizes of mRNA transcriptions for PEPCK, PC, and FBPase of dairy cows corresponded to those of rats, except that there was an additional minor transcript of about 2.1 kb for FBPase in dairy cows. Concentrations of PEPCK mRNA decreased during short-term and wk 1 of long-term infusions of glucagon, when plasma insulin concentrations were increased by glucagon infusions. Ketosis-susceptible cows in which plasma insulin was decreased and fatty liver was present, may fail to up-regulate PEPCK gene expression. PEPCK mRNA concentrations were overall greater in cows with hyperketonemia and fatty liver than in normal cows. Abundance of PC mRNA was increased and FBPase abundance was not affected by 3.5-h glucagon infusions. Multiple liver biopsies during short-term glucagon infusions confirmed that glucagon stimulated initial net glycogenolysis. We conclude that PEPCK gene expression in normal cows is down-regulated potently by insulin to balance increased glycogenolysis and gluconeogenesis during glucagon infusions. We also suggest that PEPCK gene expression in cows with hyperketonemia and fatty liver may
be different from that of normal cows, but probably is not involved in the pathogenesis of bovine ketosis.

ACKNOWLEDGMENTS

The authors acknowledge Drs. R. W. Hanson, R. B. Robinson, and H. L. Fromm for provide cDNA for PEPCK, PC, and FBPase, respectively. Appreciation is extended to J. Kent for intensive care of cows, to D. Crawley and C. Achen for provision of cows, and to G. Bobe for assistance in statistical analyses.

REFERENCES


GENERAL SUMMARY

The data presented in this dissertation are from the first known long-term administration of glucagon into lactating dairy cows, which also is one of the longest intravenous administrations of exogenous glucagon in mammalian species. Short-term infusions of glucagon also were conducted for comparisons with the effects of long-term infusions of glucagon. Whole body metabolic effects as well as changes in intracellular mRNA abundance of some gluconeogenic enzymes were determined to accomplish a general objective of providing better understanding of the regulation of carbohydrate metabolism in ruminants. Metabolic regulation of gluconeogenesis is emphasized in these studies because ruminants depend on continuous gluconeogenesis to supply glucose.

It was found that glucagon infusions at pharmacological doses stimulated an initial net glycogenolysis in the liver. The initial glycogenolysis was demonstrated clearly in the study of long-term infusions of glucagon by changes of liver glycogen, which decreased by d 2 during infusions, but was restored completely by d 7 of infusions, and then continued to increase markedly, even through 3 d postinfusion. These changes of liver glycogen reflect the regulation of liver glycogen metabolism by changes in hormonal and metabolic environments caused by long-term infusions of glucagon. Glucagon at high concentrations potently increased initial net glycogenolysis, but further net breakdown of glycogen probably was blocked by increased endogenous insulin. Then, net glycogen synthesis was increased, probably by increased substrate availability caused by increased gluconeogenesis during
glucagon infusions. In addition, multiple biopsies during short-term infusions of glucagon confirmed that glucagon stimulated a net hepatic glycogenolysis during the first few hours.

Although gluconeogenic rates and hepatic glucose output were not determined directly in our studies, increased gluconeogenesis caused by glucagon in normal cows was suggested indirectly by the three reasons. First, because daily lactose yield was not changed and daily milk yield was decreased slightly by glucagon infusions, glucose utilization for milk production may have decreased very slightly. However, glucose utilization in muscle and adipose tissue could have been increased by increased concentrations of plasma insulin during wk 1 of glucagon infusions and by constantly increased concentrations of plasma glucose. Therefore, total glucose utilization by peripheral tissues could have increased, especially in wk 1 of glucagon infusion. Second, glucose that was absorbed from the gastrointestinal tract and absorbed gluconeogenic substrates could not have been increased by glucagon infusions because feed intakes did not increase as occurred in normal control cows. Third, liver glycogen had been restored completely by d 7 of glucagon infusions and continued to increase markedly thereafter. Based upon these reasons, hepatic glucose output must have increased to provide additional glucose for possible increases in glucose utilization and to maintain increases in plasma glucose concentrations. Thus, the increased hepatic glucose output must have been from an initial net glycogenolysis and later from increased gluconeogenesis. Milk protein yield significantly decreased during infusions, which implied decreased availability of substrates for milk protein synthesis in the mammary tissue and also suggested that a shift of amino acids from protein synthesis to hepatic gluconeogenesis occurred.
While glucagon elevated carbohydrate status, glucagon infusions into normal cows decreased mRNA concentrations for the key rate-determining gluconeogenic enzyme, PEPCK. These decreases were especially apparent in the short-term study where PEPCK mRNA decreased 41% after 3.5 h of glucagon infusions, whereas PEPCK mRNA in control cows increased 31% at the same time. The decreases in PEPCK mRNA is explained by the stimulation of endogenous insulin secretion during infusions of exogenous glucagon. Long-term glucagon infusions decreased PEPCK mRNA in wk 1 of infusions, while plasma insulin increased. PEPCK mRNA concentrations were inversely associated with plasma insulin concentrations. We conclude that PEPCK mRNA expressions was down-regulated by endogenous insulin during glucagon infusions.

We also found that short-term infusions of glucagon increased the abundance of PC mRNA, which is consistent with the findings by others that glucagon increased activity of PC and increased pyruvate carboxylation, thereby increasing the flux of OAA through PEPCK for gluconeogenesis. Others have shown that insulin potently inhibited gluconeogenesis. Our data suggest that decreases in PEPCK mRNA may reflect sensitive hormonal regulation of the PEPCK gene to offset increased net glycogenolysis and gluconeogenesis during glucagon infusions. We also showed that short-term glucagon infusions did not influence FBPase mRNA abundance and that the sizes of mRNA the major transcripts for PEPCK, PC, and FBPase of dairy cows corresponded to those of rats, except that there was an additional minor transcript for FBPase in dairy cows.

Metabolic characteristics of ketosis-susceptible cows differed greatly from those of normal cows. We used the FRBD protocol to induce ketosis. Cows were made obese by
overfeeding during the dry period, and they had fatty liver, decreased hepatic glycogen, and decreased plasma glucose during the immediate postpartal period. These cows were susceptible to ketosis, and some later developed a clinic ketosis caused by FRBD. Although plasma insulin concentrations in ketosis-susceptible cows at d 7 postpartum were significantly less than those of normal cows. PEPCK mRNA concentrations were similar between groups, which may suggest that ketosis-susceptible cows can not up-regulate PEPCK gene expression. Also, long-term glucagon infusions did not affect concentrations of PEPCK mRNA and plasma insulin in susceptible cows. However, PEPCK mRNA concentrations were greater overall in cows with hyperketonemia and fatty liver than in normal cows. Also, plasma glucose concentrations in the ketosis-susceptible cows were changed significantly both by glucagon infusions and by FRBD feeding. Therefore, the data suggest that expression of the PEPCK gene probably is not involved in the pathogenesis of bovine ketosis.

With respect to lipid metabolism, concentrations of plasma NEFA did not change in the long-term glucagon infusions and decreased in the short-term infusions of glucagon. It can not be concluded from these results whether glucagon is lipolytic in ruminants because insulin was involved and because it is not known whether NEFA reesterification and oxidation were affected by glucagon infusions. Also, the effect of glucagon infusions on export of hepatic lipids and TAG in normal early lactation cows was not conclusive, and warrants further study in susceptible cows.

Further studies are warranted to further refine understanding of the effects of glucagon infusions on glucose metabolism, as well as, on hormonal and dietary regulation of
gluconeogenic enzymes in dairy cows. By taking advantage of multiple blood samples and liver biopsies, regulation of carbohydrate metabolism by exogenous glucagon was studied in lactating cows in the current study. Other infusion techniques can be used to study more specific effects of glucagon or insulin. For example, a somatostatin clamp could be used to eliminate effects of endogenous glucagon and insulin, so that plasma concentrations of glucagon or insulin can be controlled more specifically. Also, a hyperinsulinemic euglycemic clamp could be used to study specific effects of insulin in vivo. Under well-controlled conditions, in vivo regulation of gene expression of gluconeogenic enzymes can be determined more specifically for glucagon, insulin, and even glucose. Also, isotopic kinetic techniques sometimes are used to quantitatively study hormonal effects on metabolic regulation. The combination of genetic and isotopic techniques can provide new approaches for metabolic studies in both nonruminants and ruminants.

Another aspect that warrants further study is dietary regulation of gene expression. Gluconeogenesis occurs continuously in ruminant liver, and substrate supply is a major regulator of gluconeogenesis, but the mechanisms for this regulation by substrate supply are not understood. Our idea of down-regulation of PEPCK mRNA by insulin to offset increased glycogenolysis and gluconeogenesis caused by glucagon agrees well with Bassett’s discussion (Bassett, J. M. 1978. Proc. Nutr. Soc. 37:273-280) that insulin increased and inhibited gluconeogenesis to balance the high gluconeogenesis in the early postpartum period. Thus, it would be informative to study regulation of PEPCK gene expression by dietary factors such as feeding-fasting-refeeding and alterations of diet composition in ruminants.
Two subclones for rat PEPCK cDNA, PCK.L11 and PCK.L15, were derived from a full-length PEPCK mRNA clone, PCK10 (provided by Dr. R. W. Hanson, Case Western University, Cleveland, OH), and they were used as templates for making riboprobe and single strand DNA (ssDNA), respectively. Sequencing of PCK.L11 and PCK.L15 was performed to determine the precise size, orientation of inserts, and the vector used. By automatic sequencing at the Iowa State University DNA Sequencing Facility, PCK.L11 was sequenced by using the reverse universal primer and PCK.L15 was sequenced by using the forward universal primer. It was also determined that the sequences from the opposite ends in the two subclones were identical. PCK.L11 could not be sequenced by universal primer and PCK.L15 could not be sequenced by reverse primer because there were poly G sequences at one end of both subclones. Therefore, by using a manual DNA sequencing kit (USB Sequencing Version 2.0, United State Biochemical Corporation, Cleveland, OH), PCK.L11 was sequenced by universal primer. By comparing with PEPCK sequences in DNA databases from GCG (Genetics Computation Group), it was finally shown that PCK.L11 was 1405 bp long and ranged from the middle of exon 1 to the middle of exon 8 of the PEPCK gene. The length of PCK.L11 was confirmed by digesting the cDNA with Pst I. Also, it was known that the inserts were cloned in pBluescript KS+.
I. Procedures

Supplies and Equipment:

1. Water bath set at 72° C
2. Water bath set at 37° C
3. Microcentrifuge (at room temperature or 4° C)
4. Beckman J-21 C Centrifuge with microfuge tube adapters
5. Sterile 1.5 ml microcentrifuge tubes
6. Various-sized pipettes with sterilized tips
7. -20° C freezer (for storage of cDNA)
8. -70° C freezer (for storage of mRNA and cDNA)
9. GF/C filters
10. Scintillation counter (with capability of counting [35S])
11. Vortex unit

Solutions and Reagents:

1. [35S]-labeled cRNA probe
2. mRNA samples
3. PEPCK ssDNA
4. Ice-cold 75% Ethanol
5. Ice-cold 95% Ethanol
6. Ice-cold Absolute Ethanol
7. TE 8
8. RNase-free water (in kit or DEPC-water)
9. 3M Sodium Acetate, pH 5.2
10. 5M Sodium Chloride
11. 0.5M EDTA
12. 2M Tris, pH 7.5
13. Formamide
14. 20% SDS
15. 0.1M Dithiothreitol
16. Melting Bath Oil
17. RNase Buffer
18. RNase salts
19. 6M TCA (stored at 4° C)
20. 5% TCA + 20 mM sodium pyrophosphate
21. Non-toxic Scintillation Cocktail
**Methodology:**

1. Pellet the cRNA at 4°C at 12,000 x g in a microcentrifuge for 15 minutes.
2. Remove ethanol and add 0.5 ml ice-cold 75% ethanol. Vortex briefly.
3. Centrifuge as in Step 1 for 10 minutes.
4. Air-dry pellets. Dissolve cRNA in 100 µl of DEPC-water.
5. Count cpm of 1 µl of cRNA probe. Calculate the amount of cRNA probe used for a total 2,000,000 counts in the one ml of hybridization mixture, and use 20 µl of the mixture that should have 40,000 cpm for each tube.
6. Dissolve ssDNA in TE 8 and quantitate ssDNA spectrophotometrically at 260 nm. Then dilute ssDNA into a concentration of 100 pg/µl. Use various amounts of ssDNA to make standards. For example, use 0, 100, 300, 500, 700, 900, and 1200 pg of ssDNA. Add DEPC-water to each tube to make a total of 20 µl of standard or samples. Measure all standards and samples in duplicate.
7. Prepare your samples of total RNA after RNA isolation. Usually, 5 µg of total RNA was used for each sample.
8. Prepare 1 ml of hybridization mixture: mix well.
10. Add 20 µl hybridization mixture to each tube of ssDNA or mRNA sample. Mix well.
11. Overlay each sample with 2 drops of melting bath oil. Centrifuge briefly.
12. Incubate overnight in a 72°C water bath.
13. Cool samples to room temperature for 15 minutes.
14. Add 1 ml RNase Buffer to each tube. Vortex briefly. Incubate for 45 minutes at 37°C.
15. Add 0.1 ml 6 M (100%) TCA. Vortex Briefly. Incubate on ice for 1 hour.
16. Filter samples under vacuum through 5% TCA + 20 mM sodium pyrophosphate (NaPPi)-wetted GF/C filters. Then, rinse tubes with 1 ml of 5% TCA + 20 mM NaPPi and filter. Finally, rinse tubes with 1 ml ice-cold 95% ethanol and filter. Air-dry filters.
17. Count all filters for 2 minutes with 8 ml of scintillation cocktail.

**Buffer Preparations:**

Concentrated Hybridization mixture (prepare just before use)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M NaCl</td>
<td>240 µl</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>16 µl</td>
</tr>
<tr>
<td>2M Tris, pH 7.5</td>
<td>20 µl</td>
</tr>
<tr>
<td>Formamide</td>
<td>500 µl</td>
</tr>
<tr>
<td>20% SDS</td>
<td>10 µl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>200 µl</td>
</tr>
</tbody>
</table>
cRNA probe  
DEPC-water  

vol. from #5  
to make 1 ml

1 ml

Ribonuclease Buffer (store at 4° C)

10× RNase salts  10 ml
Herring sperm DNA (2.0µg/ml)  5 ml
DEPC-water  85 ml
Ribonuclease A (10µg/ml)  400 µl
Ribonuclease T1 (5000,000 U/ml)  50 µl

100 ml

RNase Salts (autoclave and store at room temperature)

5 M NaCl  60 ml
2 M Tris, pH 7.5  5 ml
0.5 M EDTA  10 ml
DEPC-water  25 ml

100 ml

II. Validation

The principles of solution hybridization for quantifying PEPCK mRNA are described in Paper Two. Three criteria verified the accuracy of the method. 1) The assay was repeatable with very small intra-assay variation. The coefficient of variation (C.V.) of one sample measured five times within an assay was 2.8%. The C.V. for another sample measured five times between five assays is 9.8%. All samples in our assays were measured in duplicate, and the average C.V. for duplicate samples in an assay is 4.4%. 2) The assay is linear. The average square of the regression coefficient (r²) for standard curves in a total of 24 assays that we did is 0.997. 3) In our assays, the cpm for all samples were corrected by
background cpm, which were obtained by adding water rather than samples or standards to the tubes. The average background cpm in our assays were 387 ± 198, which is about 7.6% of the average cpm of the samples. It was found that the background levels are related to a) the amounts of radioactivity of riboprobes added to each tube, and b) the specific activity of \([^{35}S]\)-UTP used for making riboprobes. Lower background counts were obtained by using \([^{35}S]\)-UTP with specific activity > 1,000 than \([^{35}S]\)-UTP with specific activity > 400.

It also should be pointed out that this method is not without disadvantages. Three points should be emphasized. First, the inter-assay C.V. tended to be great; so it is necessary to carefully control the assay conditions, especially the amounts of radioactivity of riboprobes added to each tube. Second, a major issue when working with RNA is to prevent degradation. The sample should be analyzed as soon as possible, and mRNA samples were stored in 95% ethanol at -80 °C to prevent degradation. Third, in our assays, a low-degree of nonspecific binding may have existed in the samples. In the RNase protection assay, which is similar to solution hybridization, this problem is prevented by capturing specific hybridization after electrophoresis of the hybridized samples in solution.
APPENDIX C. PEPCK mRNA EXPRESSION IN HEPG2 CELLS AND RATS

Materials and Methods

The objective was to study acute regulation of PEPCK mRNA in both cell lines and in diabetic rats for working out techniques and for demonstration of PEPCK mRNA regulation by cAMP, insulin, diabetes, fasting and glucose. In the HepG2 cell experiment, hepatoma cells, HepG2, were thawed from liquid N storage and cultured at 37 °C in Dulbecco’s modified eagle medium (DMEM) to confluence. The cells were then subcultured in Corning polystyrene dishes (100 x 20 mm) to about 90% confluence. Later, the cells were cultured in serum-free DMEM medium for about 24 h more. Finally, HepG2 cells were treated with either 8-(4-chlorophorylthio)-cAMP [8-(4-CPT)-cAMP] or insulin for 1 h, or at first with cAMP for 0.5 h, then with insulin for another 0.5 h. Total RNA was isolated, and PEPCK mRNA was analyzed by Northern blotting.

All rats for the study with diabetic rats were from a classroom demonstration of the glucose tolerance test. Three days before treatment, all Sprague-Dawley rats were fasted for 24 h. The next day, half of the rats were injected intraperitoneally with streptozotocin (50 mg/kg BW). The streptozotocin was dissolved in cold 0.05 M citrate buffer, pH 4.5 (the half-life of streptozotocin in this solution is about 2 min). Then, all rats were fasted for 16 h just before two normal and two diabetic rats were administrated a bolus of glucose (3 gm/kg BW) orally as a 20% solution by using a gastric tube. These rats were decapitated 2 h later. Another two normal and two diabetic rats which served as zero time controls were decapitated without receiving glucose. Blood samples were collected and serum glucose
concentrations were measured by the glucose oxidase method. Total RNA was isolated from liver samples, and PEPCK mRNA was measured by both Northern blotting and solution hybridization.

Results and Discussion

PEPCK mRNA expressed in HepG2 cells was increased acutely by addition of 8-(4-CPT)-cAMP and decreased greatly by insulin (Figure 2). It seemed that within the treatment time, insulin did not overcome cAMP to inhibit PEPCK mRNA expression (Figure 2, Lane 5). Also, only one major transcript of PEPCK mRNA was detected from the bovine liver, and its size, 2.8 kb, was the same as that of other species.

Figure 1. Northern blotting of PEPCK mRNA regulated by cAMP and insulin in HepG2 cells. HepG2 cells were either untreated (Lane 1) or incubated with 0.1 mM 8-(4-chlorophorylthio)-cAMP for 1 h (Lanes 2 and 3), 5 nM insulin for 1 h (Lane 4), or first 0.1 mM 8-(4-chlorophorylthio)-cAMP for 30 min, then 5 nM insulin for 30 min (Lane 5). Bovine samples (Lane 6, 7, 8) in which 20 μg of total RNA, twice the amount of rat samples, were loaded in each lane.
PEPCK mRNA in fasting normal rats was 8-fold greater than in fasting normal rats 2 h after glucose injections, and was about the same as in fasting diabetic rats (Figure 3). Serum glucose in diabetic rats 2 h after glucose injections was 6-fold greater than in fasting diabetic rats, and PEPCK mRNA decreased by 62% in fasting diabetic rats 2 h after glucose injections. The result of PEPCK mRNA determined by solution hybridization was generally consistent with that determined by Northern blotting, but differences of PEPCK mRNA between treatments seemed to be greater when determined by Northern blotting (Figure 4). PEPCK mRNA in fasting normal rats 2 h after glucose injections was undetected and was greatly less in fasting diabetic rats 2 h after glucose injections than in non-injected fasting diabetic rats (Figure 4).

Figure 2. Northern blotting of PEPCK mRNA in fasted normal rats at zero time (Lanes 1 and 2), fasted normal rats 2 h after oral injection of a solution of 20% glucose (Lanes 3 and 4), fasted diabetic rats at zero time (Lanes 5 and 6), and fasted diabetic rats 2 h after oral injection of a solution of 20% glucose (Lanes 7 and 8).
Figure 3. Changes in concentrations of liver PEPCK mRNA determined by solution hybridization (■) and serum glucose (▲) in fasted normal and fasted diabetic rats in response to glucose injection. A 20% glucose solution was given orally to rats as described in "Materials and Methods", and values were obtained from fasted rats at zero time and 2 h after glucose injections.
Two reasons could explain the difference between Northern blotting and solution hybridization. First, this experiment was initiated in a classroom setting, and any delay in processing liver samples would cause degradation of PEPCK mRNA, which was shown as “tails” in Northern blots. Second, solution hybridization is a more sensitive method to measure quantities of mRNA than Northern blotting; therefore, it could have measured even partly degraded fragments of mRNA as shown in the current results. Interestingly, glucose feeding in diabetic rats caused a decrease in PEPCK mRNA, which may suggest that glucose at high concentrations inhibits PEPCK gene expression, assuming there were no increases of insulin in those rats. In summary, results from the preliminary studies demonstrated clearly that expression of PEPCK mRNA were acutely regulated by cAMP, insulin, fasting, diabetes, and glucose either directly or indirectly.
ACKNOWLEDGMENTS

The completion of this Ph. D. degree is a very important step in my professional career. It is destiny and common interests in science that brought me to study here in Ames, IA. There are too many people to list everyone for expressing my appreciation to them.

I thank Dr. J. W. Young for his constant trust, understanding, inspiration, encouragement, and kindness to me during my graduate program. The same appreciation should be given to Dr. G. L. Lindberg who is my co-major professor. He is the one who enlightened me to love and study molecular biology.

The partnership of A. R. Hippen and the assistance of G. Bobe in statistical analysis are recognized definitely. Also, appreciation is extended to the many friendly members of the Nutritional Physiological Group for their many tiny helps. The help of J. Kent and others in taking care of cows is invaluable.

I thank Dr. D. S. Lewis for his constructive suggestions for preparation of manuscripts. I also thank Drs. R. W. Hanson, R. B. Robinson, and H. J. Fromm for providing cDNA for the PEPCK, PC, and FBPase genes, respectively.

As always, I thank my parents, my wife, Chengwei, and my brothers and sister for their support and understanding, and I thank my little son, Benjamin, for his sweet smiles. Finally, I thank J. C. Robbins for guiding me in seeking the truth of God. Without God's help, everything is impossible.