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EFFECTS OF INSULIN AND THE INTERACTION BETWEEN INSULIN AND RECOMBINANT BOVINE SOMATOTROPIN ON THE PRODUCTION OF MILK AND ITS COMPONENTS AND ON IGF-I PLASMA LEVELS

By

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A Thesis Submitted to the Faculty of Graduate Studies
and Research in Partial Fulfillment of the Requirements for
the Degree of **Doctor of Philosophy**

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INSULIN AND MILK PRODUCTION

This Thesis is dedicated to those who have inspired and motivated my work,

Dear Marcelo and Dante Rafael

&

Rosine (1153), Saby (1197), Karina (1194), Mercer (1187), Deplus (1156), Marilou (1177), Valerie (1208), Lily (1258), Lark (1213, who participated in two experiments), Mouchet (1245), Rossie (1250), Edith (1268), Myriam (1072), Crisco (1113, who is still at Macdonald Farm), Beulah (1190), Joy (1254), Dalhia (1263), April (1331) and Casino (1335), the 19 cows who endured the trials from which this Thesis originates;
and to animals in general.
May science always move forward with the ultimate goal of improving human and animal welfare, on both a short- and long-term basis.

I know it is not reasonable to
expect that all people love animals. But I
look forward to the day when every individual is
aware of the value of non-human animals
and acts towards them with the due respect.

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LIST OF ABBREVIATIONS

ADF:	acid detergent fiber
BCS:	body condition score
bST:	bovine growth hormone
BW:	body weight
Ca prop:	Calcium propionate
CP:	crude protein
DIM:	days in milk
DM:	dry matter
DMI:	dry matter intake
GHRH:	growth hormone releasing hormone
IGF:	insulin-like growth factor
IGFbp:	insulin-like growth factor binding protein
LCFA:	long-chain fatty acids
NDF:	neutral detergent fiber
NEl:	net energy lactation
rbST:	recombinant bovine growth hormone
SCFA:	short-chain fatty acids
ST:	somatotropin or growth hormone
TDN:	total digestible nutrients
VFA:	volatile fatty acid

PREFACE

This dissertation has a General Introduction followed by five chapters that include articles that were published or are in the process of being published in peer-reviewed journals. The articles were included as they were sent for publication, including an abstract and a list of references at the end.

The experiments carried out during the PhD Program comprehend the body of this thesis. When the Program started, in 1995, original ideas to be explored were discussed with Dr. Elliot Block. Once the hypotheses to be tested were decided, I searched the literature to define the best approach and experimental design for each hypothesis. Then I created a scientific proposal for the field trials, which was accepted by Dr. Elliot Block and exposed to all professors at the Animal Science Department, through a seminar. I have done most of the field work myself, and coordinated all additional help needed. The field work involved selection of animals to be used, the feeding and weighing of the animals, installation and maintenance of jugular catheters, rumen fluid and intensive blood sampling, an effective and feasible on-barn hormone infusion system, designing of the ideal solutions to be infused, handling and aliquoting of all kinds of samples, to mention a few activities developed. Once samples were frozen, I have performed laboratory analysis of VFA and plasma glucose and insulin, and organized the distribution of all others to adequate locations. I then analysed the resulting data set, defining the best statistical models to be used in collaboration with Dr. Roger Cue. I was entirely responsible for the writing of all the three articles, which were then reviewed by co-authors.

The manuscripts have the contribution of different authors. In all manuscripts, the contribution of the supervisor throughout most of my PhD Program, Dr. Elliot Block, was vital. Dr. Elliot Block has provided part of the financial resource for the work and guidance in the study designs and data interpretation. Most importantly, Dr. Elliot Block has fomented the original scientific ideas that were tested throughout this Thesis work. Dr. Denis Petitclerc from Agriculture and Agri-Food Canada, who has also provided both financial support and scientific contribution to the design of the experiments and the

interpretation of data, is a co-author in all manuscripts (Chapters 2, 3 and 4) as well. Dr. Roger Cue provided statistical assistance for all manuscripts. It is relevant to mention his co-authorship on manuscripts 2 and 3 (Chapters 3 and 4), in which he participated in the experimental design and proposed new forms of statistical analysis of the data. Dr. Pierre Lacasse, from Agriculture and Agri-Food Canada, provided the fat composition analysis and participated in the interpretation of the results on manuscript 3 (Chapter 4). Finally, Dr. Xin Zhao and Brenda Allard are natural co-authors of manuscript 1 (Chapter 2), since they have participated both in the scientific discussions of the idea to be tested, and in the field work.

Therefore, besides the scientific knowledge obtained during this PhD Program, I had the opportunity to interact with researchers, both co-authors of the manuscripts and other professors of the Department of Animal Science of this University, who provided a stimulating environment, where the technical discussions were something to look forward to. This is a long-lasting lesson, which I am trying to reproduce wherever I have the opportunity to work.

C. F. M. Molento

March, 2001

ABSTRACT

The effects of insulin on milk production were tested employing two different approaches. Firstly, 12 Holstein cows were used to determine the effects of feeding calcium propionate (Ca prop) on dry matter intake (DMI) and production traits. The experimental design was a switchback with 2 treatments (Ca prop at 0 or 300 g/d). The DMI was lower when animals received Ca prop. Ca prop did not affect the yield of milk and its components; however, Ca prop increased protein content. The (acetate+butyrate)/propionate ratio in rumen fluid 2 h after feeding was lower when cows received Ca prop. Plasma insulin concentration was not different between treatments and the putative effect of propionate as an insulin secretagogue was probably related to the maintenance of insulin levels when DMI was lower. In conclusion, Ca prop is a potential feed ingredient to increase protein content in milk. The second approach consisted of intravenous infusion of insulin. A trial was designed to test the effects of insulin, recombinant bovine somatotropin (rbST) and their interaction in lactating dairy cows. Eight Holstein cows were used in a Latin Square design with 4 treatments: (1) intravenous infusion of saline, (2) infusion of saline and administration of 40 mg of rbST per day, (3) intravenous infusion of 12 mg of insulin per day coupled with glucose infusion and (4) rbST administration combined with insulin and glucose infusion. The theory that rbST causes a peripheral resistance to insulin was confirmed. Insulin infusion increased percent protein, percent casein and decreased milk urea content regardless of rbST administration. For milk yield, protein yield, casein yield, lactose percent and lactose yield, there was an interaction between insulin and rbST administration. Similarly, there was an interaction between insulin and rbST on plasma IGF-I levels. Fat yield was higher, with a higher content of long chain fatty acids, during rbST administration, regardless of insulin infusion. Insulin infusion decreased fat yield mainly through a decrease in long chain fatty acids, regardless of rbST administration. For DMI and fat percent there was a significant interaction between insulin and rbST administration. The results demonstrated that insulin interacts with bST in early lactation to improve milk protein synthesis and yield in dairy cows and that insulin and rbST do not interact in terms of their effects on milk fat yield. The negative effects of insulin on milk fat production are clearly confirmed.

RÉSUMÉ

L'effet de l'insuline sur la production laitière a été testé en employant deux protocoles différents. Dans le premier, l'effet du propionate de calcium (Ca prop) oral sur la consommation de matière sèche (CMS) et la production a été étudié avec 12 vaches Holstein. Cet essai avec inversion comportait 2 traitements (0 ou 300 g de Ca prop par jour). Le Ca prop a induit une diminution de la CMS; cependant, il a causé une augmentation du pourcentage de protéine dans le lait, tout en ayant aucun effet sur la production de lait ni sur ses composantes. Le ratio (acetate+butyrate)/propionate dans le liquide ruminal, 2 h après le repas, a été plus bas avec le traitement de Ca prop. La concentration plasmatique d'insuline n'a pas été changée par le Ca prop et l'effet potentiel du Ca prop comme un secrétagogue d'insuline s'est probablement trouvé masqué par l'ajustement du niveau d'insuline suite à une plus faible CMS. En résumé, le Ca prop est un ingrédient alimentaire potentiel pour les vaches afin d'accroître le pourcentage de protéine du lait. Le deuxième protocole employé était l'infusion intraveineuse d'insuline. L'expérience a été conçue pour tester les effets de l'insuline, de la somatotropine bovine recombinante (rbST) et d'une interaction possible entre les deux hormones sur les vaches en lactation. Huit vaches Holstein ont été utilisées dans un carré latin avec 4 traitements: (1) infusion de saline, (2) infusion de saline et injection de 40 mg de rbST par jour, (3) infusion intraveineuse de 12 mg d'insuline par jour avec infusion de glucose et (4) injection de rbST combinée avec infusion d'insuline et glucose. La théorie de l'induction d'une résistance périphérique à l'insuline par la rbST a été confirmée. L'infusion d'insuline a augmenté le pourcentage de protéine et de caséine et a diminué le pourcentage d'urée indépendamment de l'injection de rbST. Pour les productions de lait, protéine, caséine et lactose, ainsi que pour le pourcentage de lactose, il y a eu une interaction entre l' insuline et la rbST. De plus, il y a eu une interaction entre l'insuline et la rbST sur la concentration plasmatique d'IGF-I. La production de gras a été augmentée, ainsi que la teneur des acides gras à chaîne longue, pendant l'injection de rbST, indépendamment de l'infusion d'insuline. Par contre, l'infusion d'insuline a diminué la production de gras grâce à une diminution des acides gras à chaîne longue, indépendemmant de l'injection de rbST. Il y a eu une interaction entre l'insuline et la rbST pour les CMS et le pourcentage de gras. Les résultats démontrent que l'insuline interagit avec la bST pendant la lactation en augmentant la synthèse et la production de protéine et que l'interaction n'existe pas pour la production de gras. L'effet négatif de l'insuline sur la production de gras a été confirmé.

ACKNOWLEDGEMENTS

Many were the people who have made fundamental contributions to this work. Elliot Block, who accepted me as a graduate student and supported my work in many ways, Humberto Monardes, Javier Burchard, Newton Pohl Ribas, Roger Cue, Denis Petitclerc, Rodrigo de Almeida, Brenda Allard, Lisa Gloutney, Barbara Stewart, Lise Ménard, José Carreño, Etienne Tremblay, Sophie Gagnon, Jennifer Bender, Carrolyn O'Grady, John Fletcher, Paul Luimes, Xin Zhao. Many people in the department of Animal Science were not directly related to the thesis work, but left important positive lessons, especially Bruce Downey and Ian Pika. A word to thank you for the singular contribution each one of you has made to my professional life.

Difficult to put in words the participation of Dr. Paul C. Laguë, who accepted the function of Thesis supervisor after Dr Block's departure. Dr Laguë was never demanding, but always ready to help. Thank you.

On a more personal note, thanks to all dear friends I have met along the way. Ana Paula, Márcio, Patrick, Susanne, Denis, Kim, Val, Thom and the Disband, Margo and the Ste. Anne Singers.

My mother has planted in my heart the first seed regarding the value of learning. My brothers Cláudio, Marcelo and Flávio have been always present, each in their own important way. Today, Dante and Marcelo are a part of my soul. Thank you.

THESIS OFFICE STATEMENT

In accordance with the regulation of the Faculty of Graduate Studies and Research of McGill University, the following is included in this thesis.

Candidates have the option, subject to the approval of their Department, of including as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If that option is chosen, connecting text that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that is more than a mere collection of manuscripts, in other words, results of a series of papers must be integrated.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research; a comprehensive review of the literature, and a final conclusion and summary.

An additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and other, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

N.B. When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

GENERAL INTRODUCTION

Animal agriculture has been a part of the human-animal interaction for at least 10,000 years, when a distinct shift marks initial human management and the transition from hunting to herding (Zeder and Hesse, 2000). Throughout this lapse of time, humankind has used different strategies to improve production, productivity, quality and safety of the final product. Most of the strategies used for production enhancement can be grouped as being either genetic improvement, management improvement or, more recently, the use of biotechnology. In general terms, these techniques have been successful in attaining the improvements originally sought. For example, average milk production from Holstein cows registered at the Programme d'Analyse de Troupeaux Laitiers du Québec in 1999 was 9099 kg per cow per 305 days (PATLQ, 2000). This is an unbelievable number when we think that the ancestors of these cows, generations ago, produced milk with the sole purpose of feeding their young. Many times there has been controversy on the ethical aspects of this race towards ever-increasing production, as illustrated by the current and passionate discussion on biotechnology. There is no simple answer to these ethical concerns. A deep consideration of all steps involved in a certain technique, its final objectives, as well as its consequences in the broadest sense possible are the best counsellors.

How is the increase in milk production obtained possible from the point of view of the physiology of the dairy cow? And how is it explained mechanistically? The work presented here tries to elucidate a small piece of this huge puzzle. The focus is the understanding of selected hormonal mechanisms that control milk synthesis in the dairy cow. Experiments were designed to lead dairy cows to a different hormonal status, either by manipulating their diet or by injecting exogenous hormones. The hormones chosen were insulin and recombinant bovine somatotropin. The effects of treatments with these hormones, mainly regarding milk and milk component yield, are reported. It is the sincere expectation of the author that this knowledge contribute to a better understanding of the physiology of the dairy cow and that its use be guided by ethical principles, taking into

account both the sustainability of the milk production system and the aspects related to animal welfare.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The realm of biological studies has experienced a vast expansion in knowledge, which is becoming increasingly faster. Today there are groups of researchers trying to describe bodily functions in such details as to build computer models that would mimic physiological reactions (McNamara and Baldwin, 2000; Cherepanov *et al.*, 2000). These very same studies show the difficulties on the understanding of the dynamic and integrated nature of metabolic and physiological functions. Even when focussing only on one aspect, such as endocrine relationships, a contextual understanding of the hormonal control of physiological processes in mammals is a daunting task (Tucker, 2000). This concept is particularly true when the system in focus is milk production, since lactogenesis and galactopoiesis involve a homeorhetic phenomenon whereby energy use must be redirected toward the mammary gland. Evidently, energy use by non-mammary tissue must be modified as well. In other words, lactation by definition means a re-orchestration of most bodily functions, being the nervous and endocrine systems the directors in charge. They are responsible for keeping the performers, each individual organ, in tune with each other, within adequate proportions of activity, so that the final goal is attained. The multitude of molecules involved in the process is appalling. In order to grasp the details, scientists have been fragmenting the whole, delving into the description of each known molecule and its functions. In this manner, a big picture is cut into billions of tiny pieces, which can then be studied. When a significant amount of knowledge is accumulated, comes the kafkaesque attempt of restoring the big picture. This attempt, with regards to the endocrinology of lactation, has proven to be extremely difficult, as mentioned by Tucker, 2000, an experienced scientist in the area who accepted the challenge of reviewing the information available. Regardless of the magnitude of the problem that emerges due to this fragmentation, it is absolutely necessary. Only by giving attention to each particular aspect will knowledge step forward into being a more truthful representation of reality.

The work presented here is responsible for a further fragmentation of the effects of insulin and somatotropin in the regulation of mammary synthesis of milk and its

components in the dairy cow. The results present additional information on the roles of these hormones, fragmented to the level of their interaction with each other. A brief search in the literature reveals a plethora of publications regarding these hormones, with excellent review articles. To a certain degree, these review articles will be duplicated and updated here. This literature review was written with the hope of focusing and organizing knowledge so that the original information presented in the subsequent articles can be put into perspective.

1.2 INSULIN

The term insulin comes from the Latin word insula, which means island (Ferreira, 1999). This denomination is a reference to the site of synthesis and secretion of this hormone, the ß-cells, which are organized together with the α- and δ-cells as small islets in the pancreas, called islets of Langerhans (Guyton, 1997).

The discovery of insulin by Frederick Grant Banting (Banting and Best, 1922), from London, Ontario, made him a Nobel Laureate in Medicine in 1923. Suddenly diabetic patients had their prognosis reverted from rapid decline followed by death to that of an almost normal life (Guyton, 1997). Thirty-five years later, Frederick Sanger won the Nobel Prize in Chemistry for his work on the chemical structure of insulin (Sanger, 1959).

Insulin is the principal hormone associated to abundant energy availability. The anabolic actions of insulin are summarised by its ability to induce (1) hepatic and muscular glycogenesis to store energy from carbohydrate, (2) fat deposition in the adipose tissue to store energy that comes directly from fatty acids or indirectly from carbohydrate and protein, and (3) amino acid uptake by cells for protein synthesis. Simultaneously, insulin inhibits lytic processes, such as glygogenolysis and lipolysis, which would exacerbate energy availability (Guyton, 1997). Lack of insulin leads to protein depletion, marked by an increased protein catabolism and a cessation of protein synthesis. The resumption of growth in pancreactomised and hypophysectomised rats depends on ST and insulin supplementation simultaneously. Guyton, 1997, states that this

synergistic and indispensable action of both hormones for growth might depend on the stimulation of cellular uptake of different amino acids by each hormone.

Insulin promotes the entry of glucose into tissues where it can be oxidized or stored as glycogen. The uptake of glucose is enhanced through increased translocation of glucose transport units to the plasma membrane; glycogen storage is favoured via activation of glycogen synthetase and inhibition of glycogenolysis (Weekes, 1991). By diverting glucogenic nutrients to muscle, insulin reduces the amount presented to the liver, and less is available for gluconeogenesis. In addition, the ability of insulin to inhibit phosphoenolpyruvate carboxykinase gene transcription, the rate-limiting enzyme for hepatic gluconeogenesis, is well documented, including description of intracellular mediators (Sutherland *et al.*, 1998). Insulin stimulates lipogenesis and inhibits lipolysis, it also stimulates cellular uptake of amino acids and their incorporation into protein, simultaneously inhibiting proteolysis (Bassett, 1975).

Brockman, 1986, proposed that ruminants are more resistant to insulin than non-ruminants, based on a slower response of blood glucose to insulin therapy. For example, hepatic glucose output was suppressed by 50 % with 2.08-2.50 ng of insulin/ml in euglycemic sheep (Weekes *et al.*, 1983), while only 1.25 ng of insulin/ml was necessary in humans (Rizza *et al.*, 1981). The smaller response to insulin of ruminant tissues can be attributed to the pattern of feeding and the action of the rumen; both minimize surges of nutrients entering the portal vein and, as a result, decrease the need for insulin to act rapidly to move nutrients into storage.

1.2.1 Background information

1.2.1.1 Chemistry

Insulin is a polypeptide hormone of 51 amino acid residues, organized in two chains, denominated A and B, linked together by two disulfide bridges (Ganong, 1987). Cattle insulin shares 92 % homology with human insulin, having only 4 amino acids substitution.

1.2.1.2 Secretion

Insulin secretion in ruminants is stimulated by feeding and by the absorbed products of digestion; it is also modulated by cold exposure, exercise and obesity (Weekes, 1991). Insulin secretion is affected in a paracrine fashion by the somatostatin produced by the δ-cells, which inhibits both insulin and glucagon secretion (Guyton, 1997). Indeed, somatostatin seems to be a potent inhibitor of insulin secretion in dairy cows, as shown by the work of Rose *et al.*, 1998. They found plasma insulin levels reduced to nearly zero by the infusion of somatostatin. Insulin response to feeding is reduced when lactating dairy cows are fed a high-concentrate diet, either in multiple feedings in one day or as total mixed ration (Sutton *et al.*, 1988). Serum insulin levels were not changed when feeding a ration composed of 45 to 50 % grain twice or 12 times per day (French *et al.*, 1990). Insulin and insulin:glucagon ratio are decreased in lactating cows when intake is restricted (Cohick *et al.*, 1986). As lactation advances and energy balance becomes progressively more positive, insulin concentrations begin to rise (Smith *et al.*, 1976). However, Blum *et al.*, 1999, reported stable levels of insulin secretion, as well as insulin metabolic clearance rate and insulin-dependent glucose utilization, between week 9 and 19 of lactation. Lower levels of insulin are found in higher yielding cows (Herbein *et al.*, 1985). At peak lactation, however, insulin levels are comparably depressed in both high- and low-yielding cows (Sartin *et al.*, 1988). Insulin secretion in response to hyperglycemia is reduced during lactation: the mean plasma insulin increments over the baseline during experimentally induced hyperglycemia were 0.96 ng/ml in lactating cows and 4.25 in non lactating cows (Sano *et al.*, 1993). The major determinant for the stimulation of insulin secretion in ruminants is the rate of increase in volatile fatty acids concentration, being propionate the most important volatile fatty acid to act as an insulin secretagogue and butyrate playing a secondary role (Harmon, 1992). Amino acids and glucose act synergistically as insulin secretagogues, since the increase in plasma insulin which resulted from the infusion of a glucose-amino acid mix significantly exceeded that obtained by isocaloric infusions of amino acids or glucose

separately (Lemosquet *et al.*, 1997). In sheep, treatment with bST increases the rate of entry of insulin to the liver via portal vein, thereby implying increased secretion of insulin (McDowell, 1991).

1.2.1.3 Blood levels

In 1972, Trenkle reviewed plasma concentration values in cattle and reported that they ranged from 0.62 to 2.08 ng/ml. With the augmentation of data available since the seventies, there came a wide range of values reported for blood concentrations of insulin in cows, and this variation is not always easy to explain with the expected variation due to physiological and nutritional conditions of the animals. From the literature, the lowest value found is 0.31 ng/ml for cows in early lactation (De Boer *et al.*, 1985) and the highest is 3.57 ng/ml (Koprowski and Tucker, 1973), being the latter an average throughout lactation. Exacerbating the natural variance of plasma insulin between animals, physiological and nutritional state, there seems to be an array of artefacts. Factors such as differences in laboratory techniques and types of antibodies used for the radioimmunoassays impose a significant contribution to that variation. For this reason, conclusions should be drawn from comparisons within rather than between experiments.

In humans, insulin has a half-life in circulation of about 5 minutes. Although over 80 % of the circulating insulin is normally degraded by the liver and kidney, virtually all tissues have the ability to metabolise it (Ganong, 1987). In ruminants, the half-life of exogenous insulin has been measured to be between 12 and 14 minutes (Trenkle, 1972).

Plasma insulin levels and the molar insulin:glucagon ratio decrease in early lactation (Vasilatos and Wangness, 1981; Sartin *et al.*, 1988), due to a reduced responsiveness of the pancreas to insulin secretagogues (Lomax *et al.*, 1979) and possibly an increased rate of insulin degradation (Debras *et al.*, 1989).

1.2.1.4 Receptors

Insulin receptors have been described in details for adipose tissue, muscle, liver and mammary gland of ruminants (Vernon and Sasaki, 1991) and, more specifically, in mammary tissue of lactating dairy cows (Oscar *et al.*, 1986). Primary bovine mammary cells, in vitro, express insulin receptors as well (Baumrucker and Erondu, 2000). In mammary tissue, the concentration of insulin binding sites is higher during pregnancy than throughout lactation (Campbell and Baumrucker, 1986). The insulin receptor has some affinity for IGF-I, if IGF-I is present in concentrations that are an order of magnitude larger than for insulin (Prosser *et al.*, 1989).

1.2.2 Insulin and milk production

1.2.2.1 Historical perspective

Considering the timeframe of knowledge regarding insulin, it is only relatively recently that it attracted the attention of researchers working with milk production. Dairy cows intensively selected for milk production show decreased insulin blood levels, if compared to dairy cows not selected since 1964 (Bonczek *et al.*, 1988). However, a cause-effect relationship has not been proven, and the decrease in insulin could be a reflection of the exacerbation in the negative energy balance as animals became higher milk producers. On the other hand, it is clear that insulin is essential for lactation in terms of mammary gland metabolism. Insulin is required for secretory differentiation and responses to other hormones in mammary tissue from pregnant rodents (Topper and Freeman, 1980). Relatively high concentrations of insulin (5000 ng/ml) are required by mammary explants from pregnant dairy cows to achieve full secretory response to hydrocortisone and prolactin (Collier *et al.*, 1977). Thus there was a contradiction between laboratory work with the mammary gland and the field research.

Initially researchers reported a decrease in milk fat percentage due to higher levels of insulin, a theory that became known as the glucogenic-insulin theory of milk fat depression (McClymont, 1951). This theory is reported by many articles and become textbook information (Van Soest, 1994). More recently, it has been challenged. Lack of an increase in concentrations of glucose and insulin in serum of cows with the greatest decline in percentage of milk fat casts doubt on the ability of the glucogenic theory to explain milk fat depression completely (Gaynor *et al.*, 1995). Bauman and collaborators, during their hyperinsulinemic-euglycemic clamp studies, have not found a significant effect of insulin in reducing milk fat content and yield (McGuire *et al.*, 1995b; Griinari *et al.*, 1997a). However, other researches employing the clamp technique reclaimed that insulin does have a negative effect on milk fat production (Léonard and Block, 1997), although in this case hipoglycemia occurred. Therefore, from a search in the literature, it is apparent that the milk fat depression theory is still fomenting controversy. From an applied point of view, before the eighties insulin was not of great interest to the dairy industry, since it probably reduced the amount of fat produced by the cows. With the change in consumer attitude seen in the past decades, interest in insulin gained momentum, since protein became the single most important component in milk for manufacturing purposes (Hettinga, 1989). Insulin infusion to dairy cows improves the yield of milk protein (Mackle *et al.*, 1999; McGuire *et al.*, 1995b; Mackle *et al.*, 2000a; Griinari *et al.*, 1997b; Léonard and Block, 1997). During abomasal water infusion, the insulin clamp increased milk protein yields by 15 % (+128 g/d); when combined with abomasal infusion of casein plus branched-chain amino acids, milk protein yield was increased by 25 % (+213 g/d) (Mackle *et al.*, 1999). Later, the same group of researchers confirmed that the insulin clamp increased the yields of casein and whey protein both with and without supplementary amino acids (Mackle *et al.*, 2000a). In another study, cows receiving insulin plus glucose infusion produced 11 % and 14 % more milk protein than negative control animals (receiving saline infusion) and positive control animals (receiving glucose infusion), respectively (Léonard and Block, 1997).

1.2.2.2 Mechanism of action

The classic idea of insulin as an anabolic hormone is that it favours body weight gain by stimulating glucose uptake by peripheral tissues, including adipose tissue. Indeed, in a recent study with beef heifers, this physiological role of insulin was confirmed when the condition score change was positively associated with both insulin and IGF-I ($r = 0.38$, $P = 0.02$ and $r = 0.71$, $P < 0.001$, respectively) and negatively associated with ST ($r = -0.67$, $P<0.001$) (Lalman *et al.*, 2000). The consideration of this basic role of insulin in normal physiology of ruminants creates a paradox when confronted to modern literature regarding insulin's effects on increasing milk protein production. How can insulin stimulate synthesis at the mammary level if its main function is to drive nutrients to body storage? Far from solved, this question offers a challenge in terms of integrative endocrinology. Certainly, the answer relies on the understanding of the interaction amongst insulin and other metabolites, factors, and hormones, such as ST. In other words, the effects of increasing insulin concentrations in a given plasma ST range may be totally different from the effects of the same increase in plasma insulin when ST levels are of a different range.

High insulin concentrations are negatively correlated to milk production (Sartin *et al.*, 1988) and this is thought to be due to an increase in glucose use by peripheral tissues other than the mammary gland (Collier *et al.*, 1989). However, during lactation, the responsiveness of adipose tissue and skeletal muscle to insulin are altered in such a way that nutrients are diverted to the mammary gland at the expense of tissue deposition (Vernon, 1989). Somatotropin blocks the ability of insulin to increase lipogenesis and activate acetyl-CoA carboxylase in adipose tissue from lactating cows and sheep (Vernon and Sasaki, 1991). The factors leading to the altered tissue responsiveness to insulin during lactation have not been fully resolved, but ST is implicated. Thus, during lactation there is an increase in glucose sparing by peripheral tissues because of both the insulin responsiveness to glucose being reduced (Sano *et al.*, 1993) and the resistance of peripheral tissues to insulin being increased (Vernon and Sasaki, 1991). The combination

of these results in hyperinsulinemia and hyperglycemia, the latter making large quantities of glucose available for mammocytes to synthesize lactose (Burton *et al.*, 1994).

In order to better understand the effects of insulin in the control of ruminant metabolism, researchers proceeded with administration of exogenous insulin. Insulin treatment of normal sheep decreased plasma free fatty acids (West and Passey, 1967). Long-term insulin injections also depressed lipolytic activities in dairy cows adipose cells (Yang and Baldwin, 1973). The main difficulty of in vivo trials with insulin injections was the consequent hypoglycemia. This experimental problem was overcome with the simultaneous injection of insulin and glucose, a technique denominated hyperinsulinemic euglycemic clamp. In a 28 h hyperinsulinemic euglycemic clamp in Japanese Shorthorn cows, the plasma insulin plateau concentrations in lactating and late pregnant cows were lower than those in non-lactating non-pregnant cows, even though insulin was infused identically on a body yield basis for each cow (Sano *et al.*, 1991). This suggests that there are differences in plasma insulin clearance among cows in different physiological states, as proposed by earlier authors (Debras et al., 1989). Plasma concentrations of IGF-I were increased (Léonard and Block, 1997; Mackle *et al.*, 2000a) and IGF binding protein 2 (IGFbp2) was decreased during the insulin clamp (McGuire *et al.*, 1995; Mackle *et al.*, 2000a).

Treatment with rbST alters the response of tissues to insulin so that the glucose response to an insulin challenge is reduced (Bauman *et al.*, 1989b; Boyd and Bauman, 1989). With the use of rbST, two patterns of hyperglycemia and hyperinsulinemia have been observed: short-term (McGuire *et al.* 1992) and chronic (Peel and Bauman, 1987; Gallo and Block, 1990b; McDowell, 1991; McGuire *et al.*, 1992) increases. If plane of nutrition is adequate, elevated glucose and insulin could provide an up-regulation of hepatic high-affinity ST receptors, intensifying ST's signal for IGF-I synthesis in the liver (Burton *et al.*, 1994). Besides, it has been shown that ST injection leads to increases in blood levels of prolactin and thyroxine, as well as insulin (Bines *et al.*, 1980).

In the mammary gland, glucose uptake is essentially dependent upon glucose concentration gradients, and is independent of insulin blood levels (Laarveld *et al.*, 1981). The influence of insulin on the uptake of amino acids is controversial, although the most

recent literature tends to point to a positive role of insulin in this process. Some authors found that the amino acid uptake by mammocytes was either unresponsive to insulin (Laarveld *et al.*, 1981) or that the response was only occasional increases in arginine extraction, with simultaneous decrease in tyrosine extraction (Tesseraud *et al.*, 1992). On the other hand, inclusion of insulin in an amino acid infusion performed at the external pudic artery tended to increase mammary uptake of amino acids, although the infusion of insulin alone tended to decrease their uptake (Metcalf *et al.*, 1991). From this finding, it is apparent that there is an interaction between the effects of insulin on mammary uptake of amino acids and the availability of amino acids to the mammary gland. Mackle *et al.*, 1999, found that plasma concentrations of essential amino acids were reduced (-33 %) during insulin clamp treatments and that the effects were most dramatic for the branched-chain amino acids (-41 %) and their keto acids (-45 %); an increased mammary uptake of these is probably responsible for at least part of the decreased plasma amino acid levels, since there were significant increases in milk protein production. Although interorgan transfer of amino acids to the mammary gland is primarily by plasma, there is an involvement of red blood cells for leucine, methionine and threonine; in the case of methionine, red blood cells accounted for 14 % of its total mammary uptake (Mackle *et al.*, 2000b). Most of the studies on amino acid uptake were done only with the plasma fraction, disregarding entirely the contribution of the red blood cells to the process. Even though arteriovenous differences of essential amino acids across the mammary gland were correlated between plasma and whole blood (Mackle *et al.*, 2000b), further research in this field is warranted. Another level of complexity is given by the fact that total uptake of all amino acids is higher than net uptake and that a large proportion of the incoming amino acid is released from the cell back to the blood (Maas *et al.*, 1998).

The local mechanism of action of insulin in terms of milk, fat and protein yields is unclear. This hormone, if administered locally using an intramammary technique, has no effects on any of these variables (Mackle *et al.*, 2000a).

Thus the effects of insulin on milk protein, which are becoming clear from many recent studies reporting a positive response, are still far from being explained mechanistically.

1.3. SOMATOTROPIN (ST)

The term somatotropin is coined from two Greek words: somato, meaning body, and tropin, which is a variation of the word trophin, meaning to nourish, to feed (Ferreira, 1999). Somatotropin is also known as growth hormone. Both denominations refer to the fact that it was first recognised as a molecule involved in the regulation of growth. As early as 1921, Evans and Long found that intraperitonial administration of fresh anterior hypophyseal substance induced characteristic effects, amongst them the stimulation of growth. Later it was demonstrated that a crude ST extract increased weight gain in rats (Evans and Simpson, 1931). After that, an array of physiological actions have been linked to this hormone, such as its ability to favour protein accretion at the expense of fat deposition (Lee and Schaffer, 1934), and its effects in increasing milk production in cows (Asimov and Krouze, 1937) and goats (Asdell, 1932). It is a consensus nowadays that ST is key for normal growth, stimulating mitotic processes in growing bones, with a significant increase on osteoblastic activity (Dickson, 1993). More specifically for the ruminant animal, ST essentiality for normal growth has been well established (Hart and Johnsson, 1986). Classic studies, which will be discussed in more detail, were conducted since the 1950s and showed that ST is essential for mammary growth both in the pubertal phase and during pregnancy (Sejrsen *et al.*, 1999).

In the past 2 decades, bST has been one of the topics of research most published on. Exemplifying this is the creation of a scientific journal dedicated to ST and IGF, entitled *Growth Hormone and IGF Research*, which first number was published in February of 1999. A considerable part of the published literature in dairy cows, though, reported on efficacy and safety studies to develop required information for registration by the governmental agencies throughout the world. Collectively, these studies established that administration of rbST to lactating dairy cows increased milk yield (Etherton and Bauman, 1998). Some studies in ruminants have proposed more mechanistic objectives, devised to elucidate the fundamental basis of rbST action in eliciting a milk production increase. The present work intends to improve knowledge in terms of rbST mechanism of action, specially regarding its interaction with insulin.

1.3.1 Background information

1.3.1.1 Chemistry

Somatotropin shares a considerable structural similarity with prolactin, and this is probably related to their overlapping effects in terms of milk production regulation. They are both protein molecules of about 22,000 Daltons, containing approximately 190 amino acids; the major differences between the two molecules, as well as amongst their variants, seem to be distributed across species (Dickson, 1993). Four variants of bovine ST have been described, consisting of a single chain of 191 or 190 amino acids from the cleavage of the N-terminal signal peptide at either of two sites, and with position 127 being filled with either leucine or valine (Bauman and Vernon, 1993). All variants are single chain peptides containing two disulfide bonds. Except for the presence of a variable number of extra amino acid residues at the N-terminal, which are a by-product of the bacterial synthesis of the hormone, recombinant bovine ST is identical to endogenous bovine ST. The potency of recombinant bovine ST is similar to that of pituitary-derived bovine ST, and these molecules share biophysical, biochemical, immunological and biological properties (Hart *et al.*, 1984; Bauman *et al.*, 1985).

1.3.1.2 Secretion

The synthesis and secretion of ST are performed by the somatotrophs, which are cells in the anterior pituitary (Daughaday, 1985). The amount to be secreted is controlled by two hypothalamic factors: a secretagogue denominated Growth Hormone Releasing Hormone (GHRH), also called growth hormone releasing factor or somatocrinin, and an inhibitory hormone denominated somatostatin. Growth Hormone Releasing Hormone and somatostatin are produced by the hypothalamus; somatostatin is also produced by the δ-cells in the pancreas islets of Langerhans. The production of these hypothalamic factors is controlled by neurotransmitters and by negative feedback of both ST and IGF-I

(Daughaday, 1985; Gluckman *et al.*, 1987). In addition to the hypothalamic factors, information from other parts of the organism participates in the coordination of ST secretion. Fluctuations in blood concentrations of glucagon, insulin, IGFs and estrogen, as well as stress and the onset of sleep have been reported as physiological stimuli for the release of ST (Rawlings and Mason, 1989). The phenomenon of sleep-related ST release seems to be restricted to higher primates (Gluckman *et al.*, 1987). As opposed to short-term infusion, long-term IGF-I administration to sheep does not alter body weight gain or carcass composition; the lack of effect of IGF-I treatment can be explained by activation of feedback mechanisms within the somatotropic axis, which lead to a reduction in ST secretion and hepatic receptor levels (Breier, 1999). Indeed, the inhibition of ST release by IGF-I is well documented. Insulin-like growth factor-I potently reduces ST release and synthesis; insulin and IGF-II mimic IGF-I action in attenuating ST release, but only at 10- to 11-fold higher concentrations (Fruchtman *et al.*, 2000).

1.3.1.3 Blood levels

Concentrations of endogenous ST in cattle reach 10-15 ng/ml in the first months of life, then decline to 2-8 ng/ml until puberty, where they increase to 15-18 ng/ml, finally settling at 5-10 ng/ml in mature lactating dairy cows (Schams *et al.*, 1989). Somatotropin level in blood is elevated during periods of nutritional deficiency in several species, including cattle (Breier *et al.*, 1986), and it is elevated in underfed compared to adequately fed lactating cows (Hart *et al.*, 1978).

The half-life of ST is about 20 to 25 min (Daughaday, 1985). Fasting increases ST half-life, reducing its turnover and metabolic clearance (Trenkle, 1976), and this might be related to the fact that as lactation progresses ST levels in blood decrease (Vasilatos and Wangness, 1981), possibly reflecting the respective changes in nutritional state. When dairy cows receive daily injections of rbST, there might be a 2- to 10-fold increase in blood total ST concentrations (Schams *et al.*, 1989). There is some evidence that endogenous secretion of ST declines in response to rbST injections (De Boer and Kennelly, 1989); this fact might be related to the increase in IGF-I due to rbST

administration. Plasma ST levels attain normality shortly after rbST treatments are terminated, suggesting that the inhibition of endogenous ST due to rbST injections is not permanent (Schams *et al.*, 1989).

1.3.1.4 Receptors

Receptors for ST have been found on the following cell types: hepatocytes, adipocytes, lymphocytes, macrophages, fibroblasts, chondrocytes, ß-islet cells and osteocytes, in a variety of species (Waters *et al.*, 1989). According to these authors, there are at least two types of bovine ST receptors, the high affinity and low affinity receptors, and the factors that regulate tissue ST receptor concentrations seem to affect only the high affinity receptor type. Higher ST concentrations obtained with rbST treatment induce an up-regulation of high affinity receptors (Waters *et al.*, 1989). The lower affinity receptor type might be correlated to ST clearance (Gluckman and Breier, 1989). An ST binding protein has been described in the goat, but it does not seem to play an important role in the variation of responses to ST treatment (Jammes *et al.*, 1996).

1.3.2 Somatotropin and milk production

1.3.2.1 Historical Perspective

Today the link between endogenous ST associated to its cascade of events and increased milk yield is clear to the point of their consideration as genetic markers for selection programs in dairy. According to Parmentier *et al.*, 1999, the somatotropic axis contains the most promising candidates in this respect, as it strongly regulates milk production. Going back in time, the basis of this knowledge relates to studies done with exogenous substances. Asimov and Krouze, 1937, published the initial report of a galactopoietic effect of anterior pituitary preparations. The galactopoietic effects were proven to be more important after peak yield and the dose-dependency of the increases in

milk yield started to be understood (Folley and Young, 1940; Folley and Young, 1945; Hutton, 1957). It was only in the late 1940s that the galactopoietic component of crude pituitary extracts used was identified as the anterior pituitary ST (Young, 1947; Cotes *et al.*, 1949). This finding motivated British scientists to attempt mass-purification of bST to alleviate food shortages during World War II; however, the extraction process was expensive and it took pituitaries from approximately 200 slaughter cows to obtain enough ST to treat one cow for one day (Burton *et al.*, 1994).

When the break-through recombinant DNA technology became available, the gene for human ST (Goeddel *et al.*, 1979) was cloned into *Escherichia coli*, followed by the cloning of bST (Seeburg *et al.*, 1983). Thus, mass production of rbST became a reality by the early 1980s. Since then, a diversity of trials was made possible. The initial trials were mostly short-term, because of the limited availability of both pituitary-derived ST as well as rbST (Peel *et al.*, 1982; Richard *et al.*, 1985; Chalupa and Galligan, 1989). It became progressively easier to obtain rbST, and long-term trials, including multiple lactation trials (Annexstad *et al.*, 1990; Léonard *et al.*, 1990b; McBride *et al.*, 1990; Gibson *et al.* 1992), appeared in the literature. An important consequence of rbST availability was its approval for commercial use in several countries, which has been generating discussions and reactions, both positive and negative, from producers, consumers, and society as a whole. By 1994 there were 15 countries in which approval of rbST for commercial use has been granted: Algeria, Brazil, Bulgaria, Czechoslovakia, India, Jamaica, Mexico, Namibia, Pakistan, Romania, Russia, South Africa, United States of America, Venezuela and Zimbabwe (Bauman *et al.*, 1994).

As one example of a non-controversial topic in scientific literature, stands the ability of rbST to increase milk production. An immense number of reports supporting a somatotropin galactopoietic effect exits in the literature, especially for the bovine gender. During lactation, higher milk yields are associated to higher blood ST levels (Vasilatos and Wangsness, 1981; Sartin *et al.*, 1985). Dairy cows intensively selected for milk production show increased ST levels, if compared to dairy cows not selected since 1964 (Bonczek *et al.*, 1988). Reports from studies with rbST administration vary from 6 to 41% increases in milk yield in Holstein cows (Burton *et al.*, 1994). In general, response

to short-term administration of rbST was variable, but an increase in milk production of 2 to 5 kg/d was observed at different stages of lactation (Peel *et al.*, 1982; Richard *et al.*, 1985, Chalupa and Galligan, 1989; Dahl *et al.*, 1991). Long-term trials have confirmed the ability of rbST to increase milk yield, establishing that rbST treatment increases the persistency of milk production (Peel *et al.*, 1989; Gallo and Block, 1990b; Schams *et al.*, 1991). Milk yield increases rapidly in response to rbST, beginning 1 or 2 days after initiation of treatment, reaches a plateau after 4 to 6 days of treatment and then decline to control levels within 5 days after the cessation of rbST treatment (French *et al.*, 1990; Stanisiewski *et al.*, 1991). The same pattern is seen with 14- and 28-day sustained-release preparations, where there is a progressive increase in milk yield over d 1 to 7, and a progressive decrease during d 9 to 14 (Bauman *et al.*, 1989a) or d 16 to 21 (Léonard *et al.*, 1990b) of the injection interval. Performance potential of the animal does not seem to have an influence on rbST effects (Sullivan *et al.*, 1988; Nytes *et al.*, 1990), although very few studies looked at rbST responses before 90 days in milk. Milking three times per day did not affect the cow's ability to respond to rbST (Galton and Samuels, 1989; Jordan *et al.*, 1991). Cross-bred dairy animals (both ½ Bos indicus x ½ Bos Taurus and ¼ Bos indicus x ¾ Bos Taurus) showed an increase in milk production in the order of 22 % (Fontes Júnior *et al.*, 1997).

Milk yield from cows treated with a sustained-release preparation of rbST tends to be cyclical in that it increases sharply during the first week of treatment to slowly return to the control level within the next 3 weeks in case of 28 day sustained-release preparation (Oldenbroek *et al.*, 1989; Léonard *et al*, 1990b; Kirchgessner *et al.*, 1991). This was also reported in cows treated with a 14 d sustained-release preparation (Bauman *et al.*, 1989a; Phipps *et al.*, 1990; Eppard *et al.*, 1991; Hartnell *et al.*, 1991; Schams *et al.*, 1991; Thomas *et al.*, 1991), although the cyclic pattern was less pronounced than in the 28 d preparation. The increase in milk production observed with rbST persists for two consecutive lactations (Léonard *et al.*, 1990b; Phipps *et al.*, 1990; Eppard *et al.*, 1991; Annexstad *et al.*, 1990; McBride *et al.*, 1990). Gibson et al., 1992, reported a decrease in response in the early part of the third consecutive lactation using daily administration of rbST.

Milk composition is not generally affected by rbST treatment unless cows are in negative energy or protein balance (Bauman *et al.*, 1985; Eppard *et al.*, 1985), when there is a trend for milk to contain higher concentrations of long chain fatty acids. When cows are injected with a sustained-release preparation, the gross composition of milk was not changed when averaged over the lactation (Léonard *et al.*, 1990a; McGuffey *et al.*, 1990). In the case of sustained-release preparations, a sawtooth pattern on both milk yield and percents of fat and protein has been observed, consistent with the release of rbST (Bauman *et al.*, 1989a; Oldenbroek *et al.*, 1989; Vérité *et al.*, 1989; Hartnell *et al.*, 1991; Kirchgessner *et al.*, 1991; Thomas *et al.*, 1991). In goats, the pattern of response to slow-release somatotropin has been well fitted by a curve containing two exponential terms, where daily relative response of treated animals was computed as a deviation of least square means of milk yield at day of interest from least square means of milk yield at day 27 of the interval (Gallo *et al.*, 1997). The impact on temporal composition of milk when sustained-released preparations of rbST are used has probably no practical consequence in terms of composition of bulk milk. Milk lactose and mineral contents are essentially not affected by rbST treatment (Eppard *et al.*, 1985; McBride *et al.*, 1988; Bauman *et al.*, 1989a; Oldenbroek *et al.*, 1989; Hartnell *et al.*, 1991), although French *et al.*, 1990, reported increased milk lactose concentrations with short-term treatment. Working with goats, Faulkner, 1999, observed that the concentrations of glucose in milk increased significantly by approximately 50 % in the period following growth hormone treatment, at a time corresponding to the increase in milk yield; concentrations of IGF-I in milk increased approximately 150 %, and were starting to decline by the time that milk yield and milk glucose concentrations were at their maximum.

Bauman *et al.*, 1999, published the biggest compilation of data regarding field results of rbST use. In their study, the response to rbST was examined in northeast USA dairy herds, from 1990 to 1998. Combining Dairy Herd Improvement (DHI) USA records and Monsanto customer files, control group (never purchased Monsanto rbST - Posilac®) and an rbST group (used on at least 50 % of cows) were identified; a total of 340 herds were involved and there were over 80,000 cows, 200,000 lactations and 2 million test-days. Assuming 100 % of cows were supplemented, response to rbST over a 305-d

lactation equalled 894 kg of milk, 27 kg of milk fat and 31 kg of milk protein. Profitability, especially for intensive farming systems, has also been reported as being positively affected by rbST administration. It was estimated that the use of rbST in New-York state dairy farms increased milk production by cow by an average of 510 kg/year and improved profitability of herds using rbST on at least 25% of cows by 120 US$/cow/year (Tauer and Knoblauch, 1997). It seems today, in view of results obtained by Bauman *et al.*, 1999, that 510 kg/year was not an overestimation of rbST influence on milk production. A positive effect of rbST use on the environmental impact of milk production has been predicted as well, since a given amount of milk can be produced with a smaller number of cows, as compared to animals not receiving rbST (Bauman *et al.*, 1994).

1.3.2.2 Mechanism of action

The mechanism of action of ST remains a puzzle (Sejrsen *et al.*, 1999). It was initially thought that ST mode of action was an enhancement of mammary lobule-alveolar development, which in turn would culminate with higher levels of milk synthesis (Asimov and Krouze, 1937). The ability of ST to increase the availability of milk precursors to the mammary gland was proposed by Hutton, in 1957. Until the late 1970s, the accepted mechanism of ST-induced increases in milk yield was the increase in blood levels of free fatty acids from an acute lipolysis of adipose tissue (Bauman and McCutcheon, 1986). This rationale was corroborated by the fact that most of the studies then employed fat and low producing cows, in short-term experiments designed with a small sample size (Bauman, 1992). Two groups of researchers, Bauman and collaborators in the United States (Bauman and Currie, 1980), and Bines, Hart and colleagues in England (Bines *et al.*, 1980), proposed a broader scope, defining mechanism of action of ST as being a homeorhetic regulation of metabolism, redirecting the partitioning and use of absorbed nutrients in favour of milk production. This rationale was later clearly proven, when McDowell *et al.*, 1987b, reported the evidence of rbST effects on partitioning of nutrients. In their study, simultaneous measurement of nutrient uptake

across the mammary gland and hind limb tissue of lactating dairy cows showed a decreased glucose uptake by the hind limb skeletal tissue and an increased uptake of NEFA by the mammary gland in response to rbST. The partitioning of nutrients theory has suited the multitude of experiments run since, where high-producing cows have had their milk yields consistently increased with the administration of rbST.

Nevertheless, increasing availability of nutrients to the mammary gland cannot be the sole factor in the response to rbST, since milk yield was not increased when cows in positive energy balance were infused with nutrients intravenously or via abomasum as much as when they were injected with rbST (Peel *et al.*, 1982; Davis and Collier, 1985). Another fact pointing to an additional mechanism is the study of amino acid extraction by the mammary gland, which shows that the increased plasma concentration of some amino acids does not explain the increased rate of extraction during rbST treatment. Hyperaminoacidemia alone only stimulated mammary leucine uptake but did not significantly modify the net metabolism of other amino acids and glucose (Tesseraud *et al.*, 1992). Increased extractions of aspartate and methionine by mammary glands in rbST treated cows were correlated positively with treatment-induced changes in arterial concentration of these amino acids; however, increased mammary extractions of arginine, leucine and lysine by rbST-treated cows were not correlated with rbST-induced changes in arterial concentrations of these amino acids (Hanigan *et al.*, 1992).

Nowadays the chronic galactopoietic effects of rbST are described mechanistically as a summation of an alteration of tissue sensitivity to homeostatic regulators (e.g. diabetogenesis), increased hepatic gluconeogenesis, changes in lipogenesis and lipolysis by adipose tissue, increased cardiac output, increased blood flow to the mammary gland, increased nutrient uptake and increased synthetic capacity of mammary secretory tissue (McBride *et al.*, 1988). There is also some evidence that ST reduces the process of involution of the mammary gland, resulting in increased persistency of milk production (Politis *et al.*, 1990). And there are the results from Kann, 1997, suggesting that mammogenesis and/or early lactogenesis are in part controlled by ST, in ewes artificially induced to lactate. This last citation shows that Asimov and

Krouze with their work from 1937 maybe were already elucidating a piece of the puzzle with their hypothesis.

An important concept to be taken into consideration when studying ST effects on lactation is the nutritional uncoupling of ST and IGF-I, meaning that during nutritional deficiencies the ability of ST to stimulate IGF-I secretion is decreased. There are many reports on the relationship between undernutrition and the uncoupling phenomenon (Breier *et al.*, 1986, 1988a, 1988b, 1989; Ronge *et al.*, 1988, 1989a, 1989b; Elsasser *et al.*, 1989). In mid lactation dairy cows, McGuire *et al.*, 1992, found no effect of dietary intake of protein or energy on basal IGF-I concentration; however, when treated with rbST, the circulating level of IGF-I increased with a significant higher response in cows receiving the diet high in both energy and protein. Moreover, only in the same group of animals, those receiving the high protein-energy diet, plasma insulin levels increased significantly in response to rbST treatment. The increased secretion of ST due to undernutrition is accompanied by the development of ST resistance at both the receptor and post-receptor levels, which in turn leads to decreased IGF-I secretion and plasma level as well as an alteration in IGFbp distribution. Changes in the number of high-affinity ST receptors were observed in restricted animals (Breier *et al.*, 1988b). Ronge *et al.*, 1988, suggested the following mechanisms for the uncoupling of ST and IGF-I: nutrient availability, sensitivity of hepatocytes to ST, and low insulin and thyroid hormone levels. Insulin was implicated in the mediation of these effects in streptozotocin-induced diabetic rats (Baxter *et al.*, 1980; Carlsson *et al.*, 1989), but these experiments involved non-homologous systems. The ST resistance developed in such a situation makes sense from a physiological point of view: it allows catabolism and puts priority on vital functions rather than growth, since nutrient-frame is restricted. Short-term GHRH administration revealed yet another mechanism of interaction between undernutrition and the somatotropic axis. When administering GHRH for 10 d, Lapierre *et al.*, 1995, found that feed restriction did not affect ST, IGF-I or milk responses to GHRH, but suppressed the increase in concentrations of insulin and IGFbp-1 and –3 following treatment with GHRH for control cows; these changes in IGFbp alter the physiological activity of plasma IGF-I.

In general, changes in dry matter intake are not observed in short-term experiments (Richard *et al.*, 1985; Dahl *et al.*, 1991). This fact led to an initial and equivocate conclusion that the improvement in performance was due to an improvement in feed digestibility or energetic efficiency. It has been shown, however, that digestibility of dry matter is not affected by exogenous rbST (Peel *et al.*, 1981; Tyrrell *et al.*, 1982; Tyrrell *et al.*, 1988). Neither digestibility nor metabolizability of the diets is affected by rbST treatment (Kirchgessner *et al.*, 1988; Tyrrell *et al.*, 1988). It has also been shown that rbST does not alter rumen fermentation, rate of passage of both liquid and solid phases, rumen microbial population and apparent digestibility of nutrients (Winsryg *et al.*, 1991). Long-term experiments show a gradual increase in feed intake, observed 8 to 10 weeks after the initiation of somatotropin treatment (Bauman *et al.*, 1985; McBride *et al.* 1988; Léonard *et al.*, 1990b; McGuffey *et al.*, 1991). Still, there are improvements in the efficiency of feed utilization for milk production during rbST treatment because of the dilution of feed costs for maintenance at a higher production level (Bauman *et al.*, 1985), and it is correct to conclude that rbST increases gross efficiency of milk production. These authors found that the net increase in energy intake observed for cows receiving 27 mg of rbST per day was 5.1 Mcal/d, which was sufficient energy to account for 7.4 kg of 3.5 % fat-corrected milk, or 73 % of the extra milk produced by cows in this group. Differences amongst trials in feed intake responses might be related to the variation in milk production responses to rbST.

Restoration of body reserves after peak lactation seems to be important for the cow to maintain her capacity to respond to rbST (Léonard *et al.*, 1990b), and if the cows are unable to maintain the increased dry matter intake observed during the rbST treatment, milk production returns to a level similar to the untreated cows (Hemken *et al.*, 1991). Gibson *et al.*, 1992, observed a 10 to 15 % depression in milk production and a similar decrease in feed efficiency when rbST treated cows were in the first 9 weeks of a third lactation that was preceeded by two lactations on rbST treatment; this depression decreased as lactation progressed and therefore the authors suggested that body condition score at the beginning of the lactation may have contributed to this effect. Similarly, Hemken *et al.*, 1991, reported that cows receiving 20.6 mg/d of rbST for a second

lactation could not maintain milk yield above that of control animals after 20 weeks of rbST treatment in the second lactation and that it was related to a decrease in dry matter intake during week 20 to 33. Adriaens *et al.*, 1992, reported an increase in milk yield of 3.3, 5.9, 1.9 and 4.2 kg/d in four consecutive lactations using 500 mg of rbST each 14 days; no explanation was given for the variation in the response to rbST, especially in the third lactation.

When in negative energy balance, cows resort to adipose tissue as a source of fatty acids for milk production, resulting in a higher milk fat percentage (McBride *et al.*, 1988). Reflecting this adipose tissue mobilization, the fatty acid composition of milk is altered to contain higher concentrations of long chain fatty acids (McBride *et al.*, 1988) as well as more unsaturated fatty acids (Eppard *et al.*, 1985). Bitman *et al.*, 1984, showed that subsequent to rbST administration milk fat content of long chain fatty acids (C17:0 to C18:3, particularly C18:1) was increased at the expense of short chain fatty acids (C4:0 to C15:0). These changes were consistent with a difference between energy balances of control (-1.6 Mcal/d) and rbST treated (-13.7 Macl/d) cows (Bitman *et al.*, 1984) and the mobilization of adipose tissue, which is composed of about 45 to 50 % C18:1 fatty acid (Christie, 1979). Eppard *et al.*, 1985, report an increase in milk fat percentage and C16:1 + C18:1 content only when rbST administration exceeded 50 IU/d and cows entered a negative energy balance.

Responses to rbST in terms of milk protein content are less marked than those of milk fat percentage. In some trials, average protein content increased slightly (Bauman *et al.*, 1989a), particularly at the end of lactation, which was attributed to the increase in energy intake. On the other hand, most researchers found no effect of rbST on milk protein content (Peel *et al.*, 1985; Lebzien *et al.*, 1989; Léonard *et al.*, 1990a; Austin *et al.*, 1991). Milk protein may be depressed if treated cows are in negative energy or protein balance (Fronk *et al.*, 1983; Eppard *et al.*, 1985; Staples *et al.*, 1988; Thomas *et al.*, 1991; Van Den Berg, 1991). Recombinant bST treatment has no effects on casein content of milk (Léonard *et al.*, 1990a; Van Den Berg, 1991). Thomas *et al.*, 1991, working with 890 cows in 15 different herds, observed increased milk fat percent and decreased milk protein percent at the initiation of bST treatment and a reversal of this

situation post-treatment in all herds; they suggested that at the conclusion of rbST treatment the dry matter intake was still higher, resulting in excess nutrient intake, which in turn could have been responsible for the observed decreased milk fat percent and increased milk protein percent.

Early in the history of ST research in dairy, it was recognised that there is a close association between its galactopoietic and anti-insulin activities (Cotes *et al.*, 1949). The diabetogenic effect of rbST is supported by the fact that its administration to lactating cows increases insulin resistance in peripheral tissues (Sechen *et al.*, 1990), inhibiting insulin-dependent glucose uptake by cells in peripheral tissues (Gluckman *et al.*, 1987). According to Hart *et al.*, 1984, diabetogenesis is an inherent property of the rbST molecule. For example, rbST reduces glucose uptake by the hind limb muscle and increases glucose uptake by the mammary gland (McDowell *et al.*, 1987b). Exogenous insulin required to maintain stable plasma concentration of glucose increased markedly in sheep rendered diabetic with alloxan when treated with rbST (Leenanuruksa and McDowell, 1988). Growth hormone releasing hormone reduced the glucose infusion rate that was required to maintain euglycemia during insulin infusions for cows in late lactation, further corroborating to the idea of an insulin resistance development when ST levels are higher (Rose *et al.*, 1996); in this study, though, no effect of GHRH on glucose infusion rate during insulin administration was found for cows in early lactation. Chronic elevation of blood IGF-I may be an important aspect of the diabetogenic and galactopoietic properties of rbST since high concentrations of IGF-I would compete with insulin for the insulin receptors in the peripheral tissues (Zapf *et al.*, 1986).

Vanderkooi *et al.*, 1995, working with rbST and GHRH treatments, found that the endogenous ST was less effective as an IGF-I secretagogue than the exogenous rbST, but still both treatments were similar in terms of milk yield response; because GHRH stimulates milk synthesis mostly through the same mechanisms as rbST, the authors concluded that IGF-I alone is not a complete indicator of the galactopoietic effects of the two hormones. It was critical for the results interpretation that this experiment (Vanderkooi *et al.*, 1995) attained the same level of plasma ST between animals receiving either rbST or GHRH. Dahl *et al.*, 1991, found a greater galactopoietic effect

for GHRH than for rbST, because the infusion of 12 mg/d of GHRH led to a plasma ST level of 16.3 ng/ml, whereas the injection of 14 mg/d of rbST led to a plasma ST level of 7.6 ng/ml; in this situation, plasma IGF-I was higher for GHRH treatment than for rbST treatment (202 ng/ml vs 116 ng/ml, respectively). The work by Davis *et al.*, 1989, also supports the concept that IGF-I alone does not explain the whole galactopoietic effect of rbST. They either injected lactating goats with rbST or infused them with recombinant human IGF-I for 3 days. Although both treatments increased plasma concentration of IGF-I similarly, milk yield increased only in the rbST-treated goats and therefore IGF-I infusion was not sufficient to mimic the effect of rbST on milk synthesis.

Recombinant bST administration leads to a decrease in lipogenesis and an increase in lipolysis. The relative importance of each process in milk production depends on energy balance, with decreased lipogenesis more important during positive energy balance and increased lipolysis during negative energy balance (Bauman *et al.*, 1988; Vernon and Flint, 1989). The combination of decreased lipogenesis and increased lipolysis leads to an elevation of non-esterified fatty acids (NEFA) as well as glycerol in blood. Peripheral tissues use NEFA as an energy source (Tyrrell *et al.*, 1988); the mammocytes use NEFA as an energy source and as precursors for the synthesis of milk fat (Hart, 1988). The question whether NEFA elevation is due to a direct specific effect of rbST on adipose tissue or indirectly driven by the negative energy balance commonly brought about by rbST administration is not resolved. Contributing to the first rationale are many reports such as those by Kronfeld, 1965, Bines and Hart, 1982, and Johnsson and Hart, 1986, who found that NEFA, insulin and glucose were increased in the circulation of somatotropin treated cows and by short-term incubation of adipose tissue in vitro. In addition, Gallo and Block, 1990a, 1990b, found chronic elevation of circulating NEFA, glucose and insulin for the 29 weeks of rbST treatment, even though cows were in negative energy balance for only the first four weeks of treatment. Serum NEFA were increased by the administration of 20 mg/d of rbST to cows in mid lactation and to dry cows, both in positive energy balance (Byatt *et al.*, 1992a, 1992b). In rats, chronic ST treatment increases the activity of hormone sensitive lipase and decreases the activities of lipoprotein lipase, acetyl-CoA carboxylase and palmitate synthetase, all of which favour

a reduction in lipid accretion by adipose tissue (Goodman, 1963; Bunyan and Greenbaum, 1965; Toshio *et al.*, 1981). In addition, ST directly inhibits glucose transport into adipocytes (Schoenle *et al.*, 1982), without the involvement of either IGF-I or IGF-II (Schoenle *et al.*, 1983). Then there are reports that NEFA increases are due to the negative energy balance. According to Sechen *et al.*, 1990, the increase in blood levels of NEFA is not acute, they gradually increase and reciprocally parallel the changes in net energy balance due to rbST treatment. Soderholm *et al.*, 1988, observed increased circulating NEFA in rbST treated cows, but this was significant only in the first 12 weeks of treatment; the concentration of NEFA was related to the dose of rbST and NEFA was elevated at periods when cows were adjusting intake to meet energy needs. Lormore *et al.*, 1990, reported similar results.

The understanding of ST influences on blood NEFA is complicated by two factors: (1) the degree of purity of the ST preparation used and (2) the cyclic effects of rbST sustained-release preparations. Acute release of NEFA with rbST administration in sheep has been attributed to processed fragments or a lipolytic intermediate of rbST (Hart *et al.*, 1984). Acute effects of rbST administration on NEFA elevation have not been found with more highly purified preparations of pituitary or recombinantly derived bST in most trials, except where treatment drove animals into a negative energy balance, in which case NEFA are chronically elevated (Bauman and McCutcheon, 1986). Sustained-release preparations of rbST have cyclic effects on energy balance, which points to the possibility of misleading averaging values. Levels of plasma NEFA were increased at 7 days but not at 14 days after injection of a 14-day sustained-release preparation (Schams *et al.*, 1991). More controversy was thrown to the issue of ST influence on plasma NEFA in 1999, with the publication by Putnam *et al.*, 1999. They report an increase in plasma glucose and a decrease in plasma NEFA with rbST administration prior to calving.

Somatotropin effects on milk yield are not uniform throughout lactation. Most researchers have administered rbST in the period beyond 60 days postpartum, when cows are typically beyond peak production and in positive energy balance. Milk yield could not be increased with exogenous pituitary extracts during the first 7 weeks of lactation (Fawns *et al.*, 1945). Bines and Hart, 1982, could not elicit an increase in milk production

with rbST at 5 weeks postpartum, although the same cows responded with a 10 to 15 % increase during treatment in months 5 and 6 of lactation. McDowell *et al.*, 1983, found an increase of only 1.6 kg/d when cows were administered rbST between 41 to 46 days postpartum. Richard *et al.*, 1985, administered rbST for two 10 d periods commencing at either 20 or 60 days postpartum. Milk yield was increased by 4.1 kg/d in the second period, and by only 2.2 kg/d in the first period. Thus, some authors report a degree of response to rbST in early lactation (Richard *et al.*, 1985; Vicini *et al*, 1991).

It was hypothesised that the lower response in early lactation was because of a higher endogenous bST (Bines and Hart, 1982) and of a negative energy balance resulting in a shortage of lactose precursors (Richard *et al.*, 1985). Response to a sustained-release rbST was also higher in mid and late lactation compared to early lactation (Léonard *et al*, 1990b; Thomas *et al.*, 1991; Sullivan *et al.*, 1992). Nevertheless, it seems that response to rbST in the period preceding peak milk production is less important and more variable than in later lactation. In the major study on rbST field responses by Bauman *et al.*, 1999, comparisons of lactation curves were used to identify where rbST response occurred in the lactation cycle; the analysis demonstrated that the responses in milk, milk fat and protein yield were minimal in the early phase of lactation, and then gradually increased until reaching a plateau in the last half of the lactation cycle. The mechanisms for such a difference are still not clear and the interaction between rbST and insulin seems to be implicated. Some researchers are trying an expansion of the traditional recommendation to use rbST commencing after peak milk yield. The available evidence demonstrates that rbST treatment stimulates mammary growth before puberty, but the data do not convincingly support the idea that the effect is translated into increased milk yield (Sejrsen *et al.*, 1999). The same authors state that rbST treatment during late pregnancy seems to stimulate both mammary growth and milk yield. Indeed cows receiving rbST starting 28 days prior to expected calving date produced 3.3 kg/d more milk than did controls across the first 42 d of lactation; the difference was 4.6 kg/d in week 6 of lactation (Putnam *et al.*, 1999). Full understanding of the role of ST in the regulation of normal mammary development requires more knowledge about the

interactions between this hormone, other systemic hormones and local factors (Sejrsen *et al.*, 1999).

Somatotropin seems to have the ability to up-regulate its own hepatic receptors, at least in sheep. Daily treatment of sheep with rbST markedly increases the number of high-affinity receptors, with no consistent change in low-affinity binding sites (Sauerwein *et al.*, 1991). The same authors showed a dose-response relationship between rbST treatment and both capacity of the high-affinity hepatic ST receptor and plasma IGF-I concentrations. This regulation of hepatic receptor number can be done rapidly because of its dynamic state associated to a short half-life of about 45 minutes (Baxter, 1985; Roupas and Herington, 1987); therefore, it can be of functional significance. The responses in terms of the stimulation of high affinity ST receptors and of blood IGF-I due to rbST administration depend on plane of nutrition, including energy and protein densities of the diet (Burton *et al.*, 1994).

In midlactation dairy cows, Newbold *et al.*, 1997, found that both exogenous bST and increased nutrient density, individually, led to elevated plasma IGF-I concentration and increased milk yield; however, only increased nutrient density induced an augmentation in the number of hepatic binding sites for bST. In well-nourished animals, elevated ST is associated with increased liver secretion of IGF-I and its binding proteins (Steele and Elsasser, 1989; Sara and Hall, 1990). Well-fed animals that respond to rbST injections with increased concentrations of blood IGF-I possess high affinity ST receptors in hepatocytes and adipocytes; under-nourished cows exhibit little or no increase in blood IGF-I concentrations in response to rbST injections and no detectable high affinity receptors (Gluckman and Breier, 1989). Thus, nutritional factors interact with endocrine factors to determine the state of the somatotropic axis; a restriction in either protein or energy in the diet can uncouple the ST-IGF-I axis and lead to little or no biological response (Burton *et al.*, 1994). Corroborating to this concept is the fact that insulin is a potent up-regulator of the high affinity ST receptor (Gluckman and Breier, 1989), leading to a more significant secretion of IGF-I in response to a given blood ST when nutrients are available. Léonard and Block, 1997, further confirmed that low concentrations of plasma insulin in early lactation limit the IGF-I response to rbST. The same rationale

applies for the variation on blood ST levels capacity to stimulate hepatic IGF-I secretion. In cultured ovine hepatocytes, availability of amino acids has been shown to have a direct role in the regulation of IGF-I secretion by rbST (Wheelhouse *et al.*, 1999); they found a strong interaction between the effects of amino acid availability and ST, such that ST increased IGF-I production by more than 2-fold in cells grown in 5-fold amino acid media but had no effect on IGF-I production by cells grown in 1- or 0.2-fold amino acid media. Wheelhouse *et al.*, 1999, reported that the observed effects on IGF-I peptide secretion were strongly associated with parallel effects at the RNA level. Although blood ST concentration is high in early lactation, target tissues may be partially refractory due to the nutritional status, supporting the uncoupling of ST-IGF-I axis, regarding the effects of under-nutrition on the efficiency of ST to stimulate liver IGF-I secretion (McGuire *et al.*, 1995a).

Glucose oxidation and turnover must be affected by rbST treatment, to provide substrate for the extra lactose synthesized. Peel and Bauman, 1987, measured a significant decrease in glucose oxidation to CO_2 from 17.4 to 12.3 %, and an increase irreversible loss of glucose of 270g/d in cows treated with rbST; this decrease in glucose oxidation could account for 30 % of the additional glucose required for lactose synthesis. More lactose precursor could be provided by increased liver gluconeogenesis from propionate or other glucogenic molecules in rbST treated cows, as shown by Pocius and Herbein, 1986. In fact, rbST treatment results in increased rates of gluconeogenesis and glucose oxidation in liver; in vivo rbST treatment increased in vitro conversion of labelled propionate and acetate to glucose more than 2-fold, and incorporation of labelled bicarbonate into glucose 5-fold (Knapp *et al.*, 1992). It must also be considered that the absolute amount of gluconeogenic precursors presented to the liver increase, since there is an increase in voluntary dry matter intake when cows receive rbST. As estimated by calculation from the injection of doubly labelled glucose infused to dairy cows and sheep, rbST significantly inhibited insulin-mediated glucose uptake, while it did not affect non-insulin mediated glucose uptake (Rose *et al.*, 1998; Rose *et al.*, 1997). Milk glucose content increases with rbST use and because glucose levels in milk reflect intracellular glucose content, these results indicate that part of the mechanism by which ST stimulates

milk production is by increasing the intracellular glucose availability for lactose synthesis (Faulkner, 1999).

Somatotropin also decreases the oxidation of amino acids (Eisemann *et al.*, 1986), which is necessary to allow for the increased milk protein synthesis during rbST treatment. Somatotropin promotes an uncoupling of nitrogenous precursors from ureagenesis and the recycling of those as glutamate bound for the periphery; this process seems to be dependent on the acidogenic action of ST which, as a consequence of promoting fatty acid utilization, yields protons required for driving hepatic glutamate efflux (Welbourne *et al.*, 1997). Therefore acid-base and nitrogen homeostasis are normally attuned to one another in part through the coordinated action of the ST axis on substrate fluxes. However, the same authors point that during feed restriction, when keto acid production is of importance as the consequence of incomplete fatty acid oxidation, the effectiveness of ST in sparing nitrogen is curtailed as glutamate (emanating from the liver) and glutamine (derived from muscle proteolysis) are directed to the kidneys, supporting ammoniogenesis: nitrogen balance is thus sacrificed for acid-base homeostasis.

Is there a direct effect of ST on the mammary gland? The following reasons support the rationale that the function of the mammary gland is not directly affected by ST: (1) addition of ST to cultured mammary explants stimulates neither cellular proliferation, nor milk protein synthesis and secretion, (2) there is no effect of close arterial infusion of ST into the mammary gland and (3) there has been no detection of ST receptors in bovine mammary tissue (Prosser and Mepham, 1989; Breier *et al.*, 1991). Controversially, there is evidence for a direct action of ST on the mammary cells: (1) ST increases mammary parenchymal proliferation in heifers as well as lactating cows, (2) ST injections to mice implanted with bovine mammary tissue resulted in substantial accumulation of α-lactalbumin as well as growth and differentiation of the implanted tissue, (3) *in vitro* experiments showing subtle effects of ST on maintenance of fat, casein and lactose synthesis in ruminant mammary tissue explants, and (4) the presence of low concentrations of ST-receptor mRNA in bovine mammary tissues (Glimm *et al.*, 1992). Thus the initial question is far from resolved.

Another level of complexity in studying ST functions is the subtle but critical differences depending on species-specific characteristics. For example, human ST shows lactogenic properties through binding to prolactin receptors in explants from bovine mammary gland, whereas neither ovine nor bovine ST had any lactogenic effect in bovine mammary gland explants (Gertler *et al.*, 1983). This report emphasizes the importance of using homologous systems when studying ST hormone-receptor interactions. There is no direct action and no binding of bST in bovine mammary tissue (Gertler *et al.*, 1983; Baumrucker, 1985b; Keys and Djiane, 1988), although mRNA for the ST receptor has been detected in mammary gland of cows (Glimm *et al.*, 1992). Besides, arterial infusion of ST into the mammary gland of lactating ewes did not increase milk production of the infused udder half (McDowell and Hart, 1984; McDowell *et al.*, 1987a). Thus, evidence points to the participation of other endocrine, paracrine and/or autocrine mediators of bST action on milk production.

Because of the rapidity of the rise and fall in the milk yield when cows are treated and removed from rbST treatment (French *et al.*, 1990; Stanisiewski *et al.*, 1991), it seems difficult to explain these changes in yield by an increase in cell number; it would be more logical to hypothesize that the productivity of existing cells is improved by rbST. An additional potential mechanism is through a reduction in the rate of early involution seen after peak milk production. This hypothesis is supported by studies reporting an improvement in persistency of milk production due to rbST use (Bauman *et al.*, 1985; Gallo and Block, 1990b). Changes in enzymatic systems in the milk in relation to rbST-treated cows would also support this putative mechanism (Politis *et al.*, 1990).

Blood flow and therefore nutrient supply to the mammary gland are both increased by rbST treatment of cows (Davis and Collier, 1985; Davis *et al.*, 1988; Fleet *et al.*, 1989; Fullerton *et al.*, 1989), sheep and goats (Mepham *et al.*, 1984; McDowell *et al.*, 1987a, 1987b; Fleet *et al.*, 1988). However, there is not enough basis to conclude that the increase in mammary blood flow is one of the causes of improvement in milk production due to rbST, since the increase in blood flow might be a consequence of increased metabolic activity of the mammary gland. Actually, a recent publication on simulation analysis of substrate utilization in the mammary gland describes the autoregulation of

local blood flow based on an energy criterion of control (Cherepanov *et al.*, 2000), pointing to a trend to believe that metabolism regulates blood flow, and not vice-versa. Recombinant bST injection increases the metabolic activity of the mammary gland, as shown by a rise in oxygen consumption (Davis *et al.*, 1988). The same authors reported that while cardiac output increased from 46.2 to 50.8 L/min, the portion of cardiac output perfusing the udder of Jersey cows increased from 14.4 (6.7 L/min) to 18.7 % (9.5 L/min) with rbST treatment. Consistent with increased cardiac output is an increase in heart rate observed in rbST-treated cows (Soderholm *et al.*, 1988). Miller *et al.*, 1991, reported that rbST does not alter extraction rates of nutrients; based on this data, the primary action of rbST in increasing milk production must result from increased blood flow rates to the udder, increases in metabolic efficiency of the udder or a combination of both.

There are several physiological levels to the regulation of ST actions (Norman and Litwack, 1987), including blood concentrations of other hormones, quantity and distribution of ST receptors in specific tissues, and intracellular signalling mechanisms. Administration of exogenous bST may override or alter some regulatory pathways normally encountered by endogenous ST (Burton *et al.*, 1994). Another important consideration is that the levels of ST attained with rbST administration probably face a different hormonal environment than the one present if these increased ST levels were a consequence of a natural secretion from the pituitary. As well, it is not known to what extent this hormonal milieu is shaping the variation in response to rbST treatment observed in the literature. One strategy to better understand this aspect is to manipulate the concentration of other hormones and inject rbST simultaneously.

1.4. INSULIN-LIKE GROWTH FACTORS (IGF)

Insulin-like growth factors are a group of protein molecules found to have insulin-like metabolic actions; later it was found that they possess a growth promoting action as well. In insulin target tissues, especially adipose tissue or isolated fat cells, IGF displays insulin-like activity, stimulating glucose transport, glucose oxidation, lipid synthesis from

glucose and lipolysis inhibition (Zapf *et al.*, 1978). Intravenous injection of IGF in rats causes a significant decrease in blood glucose, with higher potency for IGF-I than IGF-II and a preferential utilisation of glucose in striated muscle as opposed to adipose tissue (Zapf *et al.*, 1984).

The classical physiological function of IGF is the mediation of ST effects on growth promotion.

1.4.1 Background information

1.4.1.1 Chemistry

Insulin-like growth factors I and II share a 62% sequence homology; they are both single-chain molecules consisting of 70 and 67 amino acid residues, respectively, and containing three disulfide bridges each (Sara and Hall, 1990). Both molecules contain an amino-terminal B and A region separated by a short connecting C region that shows, respectively, a 43 and 41% sequence homology to proinsulin C region, eventhough IGFs and proinsulin are products of different genes (Daughaday and Rotwein, 1989). IGF-I molecules are identical in the bovine, porcine and human species, whereas in rats and mice the molecules differ by 3 and 4 amino acid residues, respectively, from the bovine/porcine/human type (Sara and Hall, 1990).

1.4.1.2 Secretion

Most tissues can synthesize IGF-I; however, the liver is the major source of circulating IGF-I (D'Ercole *et al.*, 1984). The developmental pattern of IGF-I expression in human and rat livers follows the IGF-I levels in the circulation, supporting the concept that the liver is the major source of plasma IGF-I (Sara and Hall, 1990).

A primary regulator of IGF-I gene expression in the adult seems to be ST, in the liver and also in many extra-hepatic tissues, such as heart, lung and pancreas; this regulation probably occurs at the level of transcription (Sara and Hall, 1990). There is a

coherent coordination in the expression of the IGF-I gene and the ST receptor during development, suggesting that ST receptor expression contributes to the expression of the IGF-I gene during development, in a tissue specific fashion (Shoba *et al.*, 1999). These authors report that for some of the tissues studied, the pattern of the ST receptor signalling mediators STAT-1, -3 and –5 and JAK2 expression was coordinated with the IGF-I gene expression. The fact that ST regulates IGF-I at the level of gene expression has been proved in cows specifically. When bST is injected to cows, it stimulates an increase in plasma IGF-I concentrations after several hours, suggesting an effect through stimulation of *de novo* synthesis rather than release from a storage pool (Cohick *et al.*, 1989). Bovine ST treatment increases serum IGF-I and hepatic IGF-I mRNA (Sharma *et al.*, 1994). Additionally, it is known today that IGFs are produced in multiple sites (Daughaday and Rotwein, 1989), and that they act in an autocrine and/or paracrine way to promote cell differentiation and proliferation (Isaksson *et al.*, 1987). Paracrine IGF-I synthesis is also stimulated by other growth factors and by ACTH, LH, FSH and TSH (Sara and Hall, 1990).

1.4.1.3 Blood levels

Plasma concentrations of IGF-I in humans and domestic animals are relatively stable across 24 h with no obvious diurnal rhythm due to a long half-life (Gluckman *et al.*, 1987) of up to 2 h (Drakenberg *et al.*, 1990). On the other hand, there is considerable variation on blood levels of IGF-I due to factors such as age, physiological state, interactions with other hormones and nutritional condition. Plasma concentrations are low at birth and increase postnatally concomitantly to the appearance of ST receptors in the liver. The natural basal concentration of IGF-I in bovine blood is around 100 ng/ml prior to puberty, as high as 2000 ng/ml at puberty and below 100 ng/ml in mature cows (Schams *et al.*, 1989). Vega *et al.*, 1991, reported an average serum IGF-I in cows at parturition of 24 ± 3 ng/ml. Basal circulating concentrations of IGF-I are low during early lactation and progressively increase through lactation and into the dry period (Ronge *et*

al., 1988; Sharma *et al.*, 1994; Spicer *et al.*, 1990; Vega *et al.*, 1991; Vicini *et al.*, 1991). Plasma IGF-I concentrations are affected by estrus. Plasma IGF-I increased approximately two days before behavioural estrus, and the IGF-I peak was observed in accordance with the appearance of estrus; then the elevated IGF-I levels declined to basal values 4 to 5 days after estrus in goats (Hashizume *et al.*, 2000). Administration of rbST leads to increases in blood levels of IGF-I in the order of 2- to 5-fold, depending on the rbST dose (Schams *et al.*, 1989; Davis *et al.*, 1987; Cohick *et al.*, 1989; Ronge and Blum, 1989a); consequently, there is an increase of IGF-I levels in milk as well (Prosser *et al.*, 1989; McGuire *et al.*, 1992).

It is well proven that plasma concentrations of IGF-I are dependent on nutritional status (Breier *et al.*, 1986; Gluckman *et al.*, 1987). It is important to emphasize that the nutritional status and the hormonal effects on IGF-I blood levels are intermingled. Exogenous bST treatment as well as increased nutrient density are associated with elevated plasma IGF-I concentrations and increased milk yield (Newbold *et al.*, 1997). The dominant endocrine influence of ST on plasma concentration of IGF-I is apparent as long as the animals are in a well-fed state. Elevated ST increases IGF-I in well-fed steers; this ability of ST is impaired when dry matter intake is reduced, which leads to a reduction in the number of ST receptors (Breier *et al.*, 1988a). It has been shown that dairy cows in positive energy balance show higher plasma IGF-I than cows in negative energy balance (Spicer *et al.*, 1990). Most authors found basal concentrations of IGF-I and the response of IGF-I to ST to be reduced during periods of insufficient protein and/or energy intake in heifers and steers (Ronge and Blum, 1989b; Elsasser *et al.*, 1989), even though results by Cohick *et al.*, 1986, did not follow the same pattern. Both basal IGF-I levels and the increment in plasma IGF-I after ST injection are most consistently affected by dietary protein intake (Ronge and Blum, 1989b; Elsasser *et al.*, 1989). In the rat, both ST receptor mRNA and IGF-I mRNA levels in the liver are reduced by starvation (Bornfeldt *et al.*, 1989).

1.4.1.4 Receptors

Receptors for IGFs are distributed almost ubiquitously in mammals (Sara and Hall, 1990), being the adipocytes an exception (Prosser and Mepham, 1989). There are two types of IGF receptors. The type I receptor is a glycoprotein with a molecular weight of 300 to 350 kd, consisting of two extracellular α-subunits and two transmembrane β-subunits. It is structurally and functionally similar to the insulin receptor; it binds IGF-I preferentially over IGF-II and insulin, and insulin cross-reacts only weakly and at high concentrations with this receptor (Prosser *et al.*, 1989). Type II receptor is structurally unrelated to the IGF-I and insulin receptor, being a single-chain polypeptide of 250 kd molecular weight (Sara and Hall, 1990). It has a high affinity for IGF-II, a very low affinity for IGF-I and does not cross react with insulin (Gluckman *et al.*, 1987; Prosser *et al.*, 1989). The response to IGFs and insulin in any tissue depends on the distribution of insulin, type I and type II IGF receptors in that tissue. Because of its lower potency compared to insulin in eliciting the effects in fat cells, it was postulated that IGF-I exerts its insulin-like function through the insulin receptor, both in human (Zapf *et al.*, 1981) and bovine (Etherton and Evock, 1986) adipose tissue. However, its potent insulin-like action in muscle appears to be mediated through the IGF-I receptors, since the potency ratio of IGF-I to insulin is higher in muscle than in adipose tissue (Zapf *et al.*, 1981; Sara and Hall, 1990). In mammary tissue, type I IGF receptors were first identified in human breast cells (Furlanetto and DiCarlo, 1984). Receptors for both IGF-I and IGF-II are present in bovine mammary tissue and increase in number during lactogenesis (Dehoff *et al.*, 1988). Primary bovine mammary cells express the two IGF receptor types and IGFbp-2, -3, -4, and –5 (Baumrucker and Erondu, 2000). The same authors reported that the examination of the IGF-I receptor during the mammary gland lactation cycle shows that its number declines at parturition, a change that coincides with decreases in blood levels of IGF-I; IGF-II and IGF-II receptor levels are largely unchanged.

1.4.2 Insulin-like Growth Factor and milk production

The first evidence implicating IGF-I in bovine galactopoiesis was the chronically elevated IGF-I concentrations in blood and lactating mammary tissue during periods of rbST administration (Prosser and Mepham, 1989). This idea was further supported by the demonstration of a 25 % increase in milk yield from mammary glands of goats that were treated with IGF-I by intra-arterial infusion (Prosser *et al.*, 1990). The research data showing a link between IGF and bST mode of action in terms of dairy production prompted an array of trials trying to elucidate the interrelationships between those hormones, milk production and nutrition. The goal, which is still being pursued, is to understand these relationships mechanistically, so as to be better able to manipulate them to the benefit of milk production.

Insulin-like growth factor-I also mediates ST action on mammary development. During puberty, when ST binds to its receptors in the stroma of the mammary gland, it stimulates IGF-I mRNA expression; this locally produced IGF-I causes the development of the mammary terminal end buds. This hypothesis has been proven by research using IGF-I knockout mice: treatment of these animals with IGF-I plus estradiol restored pubertal mammary development, while treatment with ST plus estradiol did not (Kleinberg *et al.*, 2000). Reduced peripubertal mammary development in heifers due to overfeeding coincides with reduced mitogenic activity of mammary tissue extracts and altered concentrations of IGF-I and IGFbp, once more proving their regulatory importance (Akers *et al.*, 2000). In this review article, information from in vitro studies convincingly demonstrates that much of the mitogenic activity of mammary extracts or serum can be attributed to IGF-I and that alterations in IGFbp-3 modulate its effectiveness.

1.4.2.1 Historical perspective

In 1957, Salmon and Daughaday suggested that the growth promoting activity of ST was mediated by a substance they named sulfation factor activity (SFA). Indeed SFA,

non-suppressible insulin-like activity (NSILA) and multiplication stimulating activity (MSA), all related structurally and evolutionary to proinsulin, were first discovered as three separate biological activities in serum (Daughaday and Rotwein, 1989), but are now recognised as a single group of substances. The term somatomedin was chosen to denote the factor that mediated the action of ST in the stimulation of somatic growth and that displayed insulin-like activity (Daughaday *et al.*, 1972). The somatomedins were later classified as insulin-like growth factor I and II, being IGF-I directly regulated by ST (Sara and Hall, 1990). It was only in the eighties that research trials commenced to report links between IGF and the galactopoietic effects of bST. When dairy cows were treated with bST, milk yield increased 17 % with a simultaneous increase of two to three-fold in plasma IGF-I concentrations (Davis and Bass, 1984). Peel *et al.*, 1985, first suggested that the increased secretion of IGF may have been responsible for the galactopoietic effect in cows chronically injected with pituitary bST.

1.4.2.2 Mechanism of action

Both IGF-I and IGF-II stimulate RNA and DNA synthesis, mitotic activity, uptake of amino acids and protein synthesis in many cell lines in culture (Rothstein, 1982). These effects are rapid and IGF-I is 5 to 7 times more potent than IGF-II and 50 times more potent than insulin, suggesting that the mediation is through the IGF receptors (Zapf *et al.*, 1984). Insulin-like growth factor-I has direct effects in bovine mammary explants, stimulating DNA synthesis and milk production (Shamay *et al.*, 1988; Baumrucker and Stemberger, 1989). DNA synthesis is stimulated by physiological concentrations of IGF-I in human (Furlanetto and DiCarlo, 1984) and bovine (Baumrucker and Stemberger, 1989) mammary cell lines through the type I IGF receptor. Insulin-like growth factor-I also induces glucose transport activity, casein synthesis and proliferation of the mammary epithelial cells (Prosser *et al.*, 1989; Zhao *et al.*, 1992).

Insulin-like growth factor-I is present in mammary tissue and increases when cows are treated with exogenous bST (Glimm *et al.*, 1988). Messenger RNA for IGF-I has been found in lactating mammary tissue, and rbST treatment resulted in an alteration

of its abundance (Glimm *et al.*, 1992). However, the work by Sharma *et al.*, 1994, resulted in no alteration of mammary IGF-I mRNA after bST administration, suggesting that systemic IGF-I levels are more responsive to bST treatment than mammary IGF-I production. Thus more research is needed before hypothesizing an alteration of the rate of IGF-I synthesis in mammocytes in response to rbST administration as an explanation for the galactopoietic effects of rbST injections. More recently, results of experiments in goats showed that changes occur in the concentrations of IGF-I in the environment of the mammary gland before changes are observed in plasma IGF-I concentrations and in milk yield, as reflected by the changes of IGF-I concentrations in milk (Faulkner, 1999). Therefore, the specific temporal pattern that should be aimed for studying the mammary responses to bST in terms of IGF-I production seems to be earlier than the natural inclination a researcher would have when designing an experiment. The trend of an early IGF-I response was also found during insulin clamp work. Mackle *et al.*, 2000a, when infusing insulin and glucose to dairy cows, reported the milk concentrations of insulin peaked on day 4 of the clamp, while IGF-I concentrations in milk peaked on day 1.

The paradoxical relation of serum IGF-I concentration and milk production over the lactation may be in part explained by the role of IGF-binding proteins and receptor numbers. Plasma IGF-I levels and liver IGF-I mRNA content are positively correlated but neither are correlated to milk yield; liver IGF-I mRNA is 44 % less in early than late lactation cows, and is increased by exogenous bST in late lactation (Sharma *et al.*, 1994). The bovine mammary gland has high affinity receptors for IGF-I, and their abundance is affected by rbST administration (Glimm *et al.*, 1992); there are indications that the number of IGF-I receptors in the mammary gland may be highest in early lactation (Hadsell *et al.*, 1990). Therefore, it seems that the variation in receptor number at the level of the mammocytes could compensate for lower circulating levels of IGF-I found in early lactation. It is also apparent that IGF binding proteins (IGFbp) may influence the role of IGF-I in the response to bST. Most IGF-I circulates bound to an IGFbp. The different forms of the IGFbp have different physiological roles, i.e. some act to extend the half-life of blood IGF-I or to deliver it to target tissues, some restrict its movement

from the vascular space making it unavailable for biological functions while some might be required to enhance biological activities of IGF-I (Sara and Hall, 1990).

Most of the circulating IGF-I is bound to IGFbp3 (Sara and Hall, 1990). In starvation, the proportion of IGF-I bound to IGFbp1 increases at the expense of IGFbp3. This results in a more rapid clearance of IGF-I from the circulation in view of the 20-fold shorter half-life of IGFbp1 (Hodgkinson *et al.*, 1987). Therefore, negative nutrient balance decreases circulating IGF-I levels through both decreased hepatic synthesis and increased clearance. Exogenous bST increases IGFbp3 (Hodgkinson *et al.*, 1991; Sharma *et al.*, 1994) and decreases IGFbp2 (Sharma *et al.*, 1994). These changes may increase the actions of IGF-I in a tissue-specific manner, since IGFbp2 inhibits IGF-I actions only in some tissues (McGuire *et al.*, 1992). In addition, it seems that IGF-I can amplify its own action on mammocytes, especially if receptor numbers are increased, as in early lactation. Insulin-like growth factor-I can stimulate the synthesis of IGFbp3 by mammocytes, and this binding protein amplifies IGF-I's mitogenic activity (Cohick, 1998); however, it is not known whether this mechanism operates in vivo. The concentrations of the predominant mammary IGFbp, which is IGFbp-3, declines in blood and milk during lactation, compared to prepartum and involution periods; time of lactation and pregnancy were the main determinants of milk but not blood IGFbp-3 levels (Baumrucker and Erondu, 2000). These authors also report that lactoferrin has the capacity to compete with IGF binding to IGFbp-3; this finding reveals a novel role for lactoferrin, suggesting that it is critically involved in the regulation of the IGF system during the involution period. More recently it has been proposed a role for IGFbp5 in the mammary gland involving the initiation of apoptosis, induced by the sequestration of IGF-I, which is an important survival factor for the mammary gland (Flint *et al.*, 2000). As well, these authors found indication that IGFbp5 interacts with α_{s2}-casein, implicating IGFbp5 in the regulation of plasminogen activation in the mammary gland. As such, IGFbp5 may play a key role in coordinating cell death and tissue remodeling processes.

Nitrogen sparing effects of ST also seem to be mediated by IGF-I. Peripheral retrieval of the glutamate usually salvaged from ureagenesis by ST requires IGF-I (Welbourne *et al.*, 1997).

HYPOTHESES

The hypotheses of the present work are:

- It is possible to alter endogenous release of insulin via diet manipulation in order to enhance rbST effects in lactating dairy cows;
- There is a significant interaction between insulin and ST for the control of milk and milk component synthesis;
- Simultaneous administration of insulin and rbST results in an enhancement of rbST actions on milk and milk component production;
- Simultaneous administration of insulin and rbST results in a significant increase in IGF-I secretion as compared to rbST or insulin alone;
- Simultaneous administration of insulin and rbST results in improved efficiency of nitrogen use, with higher milk protein synthesis and lower milk urea nitrogen;
- Simultaneous administration of insulin and rbST results in increased insulin resistance by peripheral tissues.

INITIAL STATEMENT

The following experiment was performed to test whether oral Calcium propionate, a putative secretagogue for insulin, is capable of increasing milk protein production through the alteration of endogenous release of insulin.

CHAPTER 2

Manuscript 1

Effects of Calcium Propionate on Milk Performance, Rumen Volatile Fatty Acid and Insulin Response in Dairy Cows

By

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ABSTRACT

Twelve Holstein cows, post-peak, were used to determine the effects of feeding calcium propionate (Ca prop) on DMI and production of milk, fat and protein. The experimental design was a switchback with 2 treatments (Ca prop at 0 or 300 g d^{-1}), 2 replicates of 6 cows and 3 periods each. Data were analysed using the SAS system for mixed models. The DMI was lower ($P<0.05$) at 21.3 ± 0.7 kg d^{-1} when animals received Ca prop vs 22.5 ± 0.7 kg d^{-1} for the control diet (0 g of Ca prop). No difference was observed when body weight gain or loss was compared across treatments ($P>0.05$). There was no difference between treatments ($P>0.05$) for milk production, fat content, fat production, protein production, lactose content and lactose production. Their overall means (SD) were 35.5 (1.5) L d^{-1}, 3.13 (0.17) %, 1.099 (0.068) kg d^{-1}, 1.039 (0.046) kg d^{-1}, 4.65 (0.05)% and 1.647 (0.092) kg d^{-1}, respectively. However, Ca prop increased ($P<0.05$) protein content (2.97 ± 0.06 %) vs control (2.94 ± 0.06 %). VFA concentrations in the rumen fluid showed no significant differences ($P>0.05$) between the two levels of Ca prop for any VFA tested, both at 2 h and 4 h post-feeding. Overall means (SD) were 118.7 (66.9), 50.0 (24.8), and 27.0 (11.9) mM for acetate, propionate and butyrate, respectively, 2 h after feeding. These concentrations fell to 102.8 (68.4), 43.6 (28.9) and 21.4 (11.2) mM, respectively, 4 h after feeding. There were significant differences ($P<0.05$) in the (acetate+butyrate) / propionate ratio 2 h after feeding, being 3.22 ± 0.30 (LSM ± SE) when cows received 0 g of Ca prop d^{-1} and 2.71 ± 0.29 when cows received 300 g of Ca prop d^{-1}. The same ratio calculated for 4 h after feeding was not different between treatments, the overall mean (SD) being 3.02 (0.80). There was no significant difference ($P>0.05$) in plasma glucose concentrations between treatments with LSM of 57.1 ± 1.18 mg/dl for the control diet and 57.9 ± 1.18 for the diet including 300 g of Ca prop d^{-1}. LSM for plasma insulin concentration was 161.6 ± 11.0 pmol/L for the control diet and 152.9 ± 11.0 pmol/L for the Ca prop diet ($P>0.05$). Statistical analysis of insulin levels revealed a significant interaction between treatment and hour of sample collection ($P<0.05$). None of the comparisons of interest (treatment effect on insulin levels within a given hour) showed a statistically significant difference. The putative effect of propionate

as an insulin secretagogue was probably related to the maintenance of insulin levels even when DMI was lower. In conclusion, Ca prop is a potential feed ingredient to increase protein content in milk. The difference on the (acetate+butyrate) to propionate ratio 2 h after feeding indicates that the amount of Ca prop fed significantly altered the balance between these VFA, which probably resulted in the increase in milk protein percentage. The feeding of 300 g of Ca prop d^{-1} did not cause an increase in plasma glucose levels nor in plasma insulin concentrations; the lack of an insulin response is probably due to the decrease in DMI with the Ca prop diet.

(**Key words:** Calcium propionate, insulin, dairy cows, dry matter intake)

INTRODUCTION

Nutrition offers opportunities for manipulating milk component production. Dietary protein does not reliably alter protein concentration in milk, and increases in energy intake seem to be the most reliable means of increasing milk protein concentration (Sutton, 1989). To overcome the difficulties in increasing feed intake in the high producing dairy cow, different types of feedstuff have been used to increase energy per unit of dietary dry matter, and thus to increase energy intake even when feed intake is not increased. It is also important to consider how different feed ingredients are metabolised, since some substances may be particularly interesting if they lead to a shift in homeostatic regulators that influence milk protein production. Calcium propionate (Ca prop) is a feed ingredient that may both increase energy concentration in the diet and alter homeostasis regulation in dairy cows. Higher levels of propionate in the rumen result in increased propionate concentrations in the portal vessels (Spears, 1990). The shift towards more propionate absorption may have hormonal consequences, as propionate is significantly correlated with serum insulin in dairy cows during mid lactation (r^2=73; Jenny et al., 1974). Insulin was also correlated with glucose (r^2=51), but the correlation of insulin to propionate was higher. Even then, Jenny et al. (1974) concluded that the probable mechanism through which propionate induced insulin secretion was mediated

via hepatic metabolism, through the conversion of propionate to glucose. More recent experiments point to a role of propionate as a direct secretagogue for insulin (Johnson et al., 1986) and for both insulin and glucagon (Sano et al., 1993) in sheep.

Many cows fail to produce to their genetic potential because of the lack of some key nutrient (Clark, 1975). Utilization of amino acids by the mammary gland is dependent not only on arterial amino acid concentrations, but also on mammary blood flow, transport efficiency and regulation of intracellular metabolic pathways (DePeters and Cant, 1992). Some essential amino acids that are transferred stoichiometrically to milk protein are potential limiting nutrients to milk protein production. Because none of these has shown 100% extraction from plasma, it is possible that the key nutrient is available but its transfer to milk protein might be limited by the inadequate supply of a key hormone (Mepham, 1982). Insulin has an effect on net metabolism of amino acids by the bovine mammary gland. Higher insulin levels in blood led to higher extraction rates of aspartate, isoleucine, leucine and tyrosine, presumably associated with a net increase in protein synthesis (Laarveld et al., 1981). Baumrucker (1985) concluded that both insulin and glucagon have a stimulatory effect on amino acid uptake by the mammary gland, at least for the A transport system, through which neutral amino acids are transported from blood to mammocyte cytoplasm. Besides favouring amino acid uptake, insulin has also mitogenic activity on the mammary cells (Houdebine et al., 1985). Evidence from in vivo trials confirms the participation of insulin in the regulation of milk protein synthesis by the mammary gland. McGuire et al. (1995), working with dairy cows in mid lactation, found an increase in protein yield when infusing insulin + glucose as opposed to saline infusion. In dairy cows receiving recombinant bovine somatotropin (rbST) during early lactation, insulin + glucose infusion increased milk protein content and kilograms of milk protein produced d^{-1}, when compared to saline infusion (Léonard et al., 1997).

Propionate is a potential feed supplement to improve feed conversion to milk protein. The objective of this trial was to evaluate the effects of feeding Ca prop to lactating dairy cows on their DMI and production of milk, fat and protein, assessing whether these

effects could be explained via an increase in plasma insulin.

MATERIALS AND METHODS

Preliminary work

A preliminary trial was conducted to determine the quantity of Ca prop that could be fed to lactating dairy cows without decreasing significantly their DMI. Four dairy cows in early lactation (ranging from 16 to 76 days in milk; 45 ± 26 DIM) were randomly assigned to one of the following treatments: (1) 0 g (control), (2) 200 g, (3) 350 g and (4) 500 g of Ca prop d^{-1}, mixed in a diet formulated according to NRC requirements (1989). The experiment was designed as a Latin Square with 4 periods of 7 days each. The guidelines of the Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, 1993) were observed for all procedures involving the animals, for both the preliminary work and the main trial.

Main trial

Animals and treatments

Twelve multiparous Holstein cows from the Macdonald Teaching and Research Complex, post-peak (ranging from 43 to 91 days in milk; 64 ± 14 DIM), were used in a switchback design with 2 treatments (Ca prop at 0 or 300 g/d), 2 replicates of 6 cows and 3 periods of 14 days each.

Management and measurements

For each period, days 1 to 3 were considered the adaptation phase (animals received treatments, were weighed and scored for body condition), days 4 to 10 were considered the measurement phase, when most of the measurements were made, and days 11 to 14

were a resting phase (all animals received the same diet without Ca prop, were weighed and scored for body condition) (Figure 1).

The cows were housed in an artificially lighted and ventilated tie-stall barn with separated mangers that allowed for the measurement of individual feed intake. All animals received the same basal diet in the form of a total mixed ration (TMR) with a 40:60 ratio of haylage (alfalfa and timothy) + corn silage to grains, formulated according to the requirements of the National Research Council (1989) and fed at around 110 % of their *ad libitum* intake, in 2 meals per day. During the measurement phase, feed offered and feed refused were weighed daily. Samples of the TMR were collected on days 3 and 11 of each period, immediately before and after the measurement phases; these samples were analysed using traditional wet chemistry procedures for crude protein (method of Kjeldahl; A.O.A.C., 1985), acid detergent fiber (method of Van Soest, 1973), and neutral detergent fiber (method of Goering and Van Soest, 1970). Ether extract, calcium, phosphorus and ash were determined according to A.O.A.C (1985) procedures. Dry matter was determined using a forced draft equipment at 100°C (Koster Crop Tester, Cleveland, Ohio). Table 1 shows the average composition of the TMR throughout the trial before the addition of Ca prop; individual sample composition is not shown since variation was small. Animals receiving 300 g of Ca prop were offered 1.5 Mcal/d over their TMR energy intake.

During the measurement phase, milk yield was recorded daily. Milk samples from morning and afternoon milkings were taken on days 8, 9 and 10. They were analysed with an infra-red analyser by the Programme d'Analyse des Troupeaux Laitiers du Québec (PATLQ) for protein and fat contents, and for somatic cell count. Samples of rumen fluid were collected 2 and 4 hours after morning feeding on day 8, with a flexible tube inserted orally and connected to a vacuum pump. Although liable to saliva contamination, this method was preferred since it is the least invasive procedure to access rumen contents; the (acetate + butyrate) to propionate ratio should not be affected by saliva contamination. The opening of the tube was fitted with a metallic screen to prevent

the suction of solids during the procedure. The pH of all rumen fluid samples was read immediately using a pH meter. Then the samples were centrifuged (10,000 x g for 10 min) and the supernatant frozen after the addition of an internal standard. Gas chromatographic analysis (Hewlett-Packard 5710 chromatograph, Hewlett Packard, Palo Alto, CA) was performed with a 4 mm i.d. glass column (2 m) packed with SP 1220 (Supelco Chromatography Supplies Inc., Bellefonte, PA), according to the method of Erwin et al. (1961). On the last day of the measurement phase (day 10), hourly blood samples were collected for the determination of plasma levels of insulin and glucose. The animals were fitted with an indwelling catheter in the jugular vein on day 9. Samples were collected from 01:00 h to 24:00 h on day 10. Each blood sample was collected into 2 different tubes: (1) a tube with sodium fluoride and potassium oxalate for glucose analysis and (2) a tube with sodium heparin for insulin analysis. The samples were immediately put on ice and centrifuged (6,000 x g for 20 minutes) within 15 minutes. Plasma was then aliquoted and frozen.

Glucose analysis was done by automated spectrophotometry (Abbott VP Super System, Abbott Laboratories, Mississauga, Canada), using a commercial kit (Sigma Diagnostics, St. Louis, Mo, USA). Intra- and inter-assay coefficients of variation were 0.5 and 0.7 %, respectively. Insulin analysis was done by a radioimmunoassay procedure using a commercial kit (KTSP-11002, Immunocorp, Montreal, Canada); the presence of radioactive insulin-antibody complexes in the test tubes was read with a γ-counter (Pharmacia-Wallac 1282 CompuGama CS, Finland). Intra- and inter-assay coefficients of variation were 3.8 and 4.6 %, respectively.

Statistical analyses

The statistical analysis of data from the preliminary trial conducted as a Latin Square was done using proc GLM (SAS Institute, 1985). The effects of the different levels of Ca prop were assessed by model [1]. For the analysis of data from the switchback design, the proc MIXED (SAS Institute, 1989) was used. Replicate, period within replicate, treatment,

day and hour were considered fixed effects. Both the effects of cow and the time trend were included in the random statement. Day and hour were included as repeated measures. The proc MIXED was used because it allows modelling the covariance structure, which is especially important for the analysis of repeated measures data (Littell et al., 1998). Different covariance structures were tested for plasma insulin and plasma glucose; the auto regressive covariance order 1 was the best and was used for the analyses of these two variables. All other traits were analysed using the variance component structure. Two-way interactions were tested and the interaction between hour and treatment for the analysis of insulin was statistically significant; all other interactions were not significant and were dropped from the model. Most parameters were analysed by model [2], except plasma glucose and plasma insulin, which were analysed by models [3] and [4], respectively.

$$Y_{ij(k)} = \mu + Per_i + Prop_j + C_{(k)} + e_{ij(k)} \qquad [1]$$

$$Y_{ijklmn} = \mu + Rep_l + Per_i(Rep_l) + Prop_j + C_k(Rep_l) + TT_m + D_n + e_{ijklmn} \qquad [2]$$

$$Y_{ijklmo} = \mu + Rep_l + Per_i(Rep_l) + Prop_j + C_k(Rep_l) + TT_m + H_o + e_{ijklmo} \qquad [3]$$

$$Y_{ijklmo} = \mu + Rep_l + Per_i(Rep_l) + Prop_j + C_k(Rep_l) + TT_m + H_o + Prop_j{*}H_o + e_{ijklmo} \qquad [4]$$

In these models, Y is the measured parameter, μ is the overall mean, Per is the period effect, Prop is the Ca prop effect, C is the cow effect, Rep is the replicate effect, TT is the time trend effect (period as a continuous variable by cow), D is the day effect, and H is the hour effect.

RESULTS

Preliminary work

Table 2 shows least square means for DMI and production traits by treatment. There was a significant decrease in DMI when Ca prop was given at levels of 350 and 500 g d^{-1}, as compared to the control diet (22.6 and 22.1, respectively vs 24.1 kg d^{-1} for the control diet; $P<0.05$). Milk yield increased significantly when animals received 200 g of Ca prop d^{-1} (46.5 vs 44.0 kg d^{-1} for the control diet; $P<0.05$)).

Main trial

DMI, BW and BCS

Feeding 300 g of Ca prop d^{-1} caused a significant decrease in DMI (Figure 2; 21.3 ± 0.7 kg d^{-1} for Ca prop animals as opposed to 22.5 ± 0.7 kg d^{-1} for the control diet; $P<0.05$). The average BW throughout the experiment was 655 ± 68 kg; there was no effect of treatment on body weight. The BCS of the animals ranged from 1.75 to 3.75; treatment did not affect BCS.

Production traits

No significant effect of treatment was found for milk yield (Figure 2; LSM of 35.6 ± 1.6 kg of milk d^{-1} for the control diet vs 35.7 ± 1.6 for the Ca prop diet). Fat content, fat production, protein production, lactose content and lactose production were not affected by treatments (Table 3; $P>0.05$). Calcium propionate had a significant effect ($P<0.05$) on protein content (2.97 ± 0.06 % vs 2.94 ± 0.06 % for the control), as shown in Figure 3.

Rumen fluid analyses

The LSM for rumen fluid pH 2 h after feeding averaged 7.1 ± 0.1 in both treatments; the value 4 h after feeding was 7.1 ± 0.1 for the control diet and 7.0 ± 0.1 for the Ca prop diet. There was no significant difference between treatments for rumen pH ($P>0.05$). VFA concentrations in the rumen fluid showed no significant differences ($P>0.05$) between the two levels of Ca prop for any VFA tested, both at 2 h and 4 h post-feeding (Table 4). There was a significant difference ($P<0.05$) in the (acetate + butyrate) to propionate ratio 2 h after feeding, being 3.22 ± 0.30 when cows received the control diet and 2.71 ± 0.29 when cows received 300 g of Ca prop d^{-1}. The same ratio calculated 4 h after feeding was not different between treatments ($P>0.05$), being 2.94 ± 0.24 and 2.78 ± 0.20 for the control and Ca prop diets, respectively (Figure 4).

Plasma parameters

There was no difference ($P>0.05$) in plasma glucose levels between treatments; the LSM ± SE being 57.1 ± 1.2 mg/dl for the control diet and 57.9 ± 1.2 for the diet including 300 g of Ca prop d^{-1}. Statistical analysis of insulin levels revealed a significant interaction between treatment and hour of sample collection ($P<0.05$). None of the comparisons of interest (treatment effect on insulin levels within a given hour) showed a statistically significant difference (Figure 5).

DISCUSSION

Preliminary work

Conclusions from the preliminary trial served only as guidelines for the main trial, since the design was not able to explain variation to any reliable extent (r^2 varied from 0.66 for DMI to as low as 0.44 for protein yield), due in great part to the limited number of animals used. Considering the results obtained, a level of 300g of Ca prop d^{-1} was

chosen for the main trial, with the goal of maximizing effects on production traits while minimizing the undesirable decrease in DMI.

Main trial

DMI, BW and BCS

The lack of an effect of treatment on BW and BCS was expected due to the length of the experiment. DMI decreased when animals received the Ca prop diet, yet no change in either BW or BCS was seen. A trend to a decreased BW or BCS would probably not be significant due to the short time basis of this experiment. Another reason for the lack of changes in these characteristics is the fact that the addition of 300 g of Ca prop d^{-1} corresponds to an increase in the energy density of the Ca prop diet as compared to the control. The NEl of the basal diet was on average 1.60 Mcal kg^{-1}, and the DMI for the control was 22.5 kg d^{-1}, which equates to a total energy intake of 36.0 Mcal d^{-1}. For the Ca prop diet, DMI was 21.3 kg d^{-1}, which corresponds to an energy intake of 34.1 Mcal d^{-1}, to which 1.5 Mcal d^{-1} were added as Ca prop (considering Ca prop gross energy equal to NEl). Thus, the total energy intake was 35.6 Mcal d^{-1} when animals received Ca prop. Hence, the difference in energy intake was not as large as the difference in DMI, and the former might not have been important enough to induce a significant change in BW and BCS.

The decrease in DMI is explained either by the low palatability of Ca prop or by the effects of propionate on the control of DMI in ruminants. The low palatability of Ca prop was evident, and cows reacted by changing their pattern of intake. When receiving the control diet, the DMI peaked right after a meal was offered, while when animals received the Ca prop diet, the intake was more even throughout the day. Montgomery et al. (1963) and Anil et al. (1993) found a decrease in DMI during infusion of propionate in the rumen, proving that propionate plays a role in the regulation of DMI. Propionate negative feedback on DMI is carried to the central nervous system through neurons, rather than via

the circulatory system. The hepatic plexus, composed of both sympathetic (splanchnic nerve) and parasympathetic (vagus nerve) nerve fibres, seems to be the most important afferent pathway, since sectioning this plexus abolishes the ability of propionate to decrease DMI (Anil and Forbes, 1980). This neural pathway explains how propionate can feedback on DMI control, even though changes in propionate levels in the peripheral blood after feeding are not clear-cut (Forbes, 1986). The decrease in DMI observed in this trial might be a combination of the low Ca prop palatability and the negative feedback this VFA exerts on the control of DMI.

Production traits

The fact that none of the production traits were affected points to an increase in feed efficiency when animals received Ca prop, considering that DMI was lower than the control. Moreover, regardless of the decrease in DMI, Ca prop was capable of increasing milk protein percentage. The mechanism through which the increase in milk protein percentage was elicited is not clear, since the hypothesized increase in plasma insulin was not demonstrated. Either there is another mechanism involved, or the experimental design, especially regarding number of animals used and quantity of Ca prop fed, was not sensitive enough to detect an effect on insulin as being the mediator of the observed changes. Regardless of the increase in protein percentage in milk, protein yield (kg d^{-1}) did not change, following the same pattern as milk yield.

Rumen fluid analyses

Rumen fluid pH values observed were higher than the expected values. In general, rumen pH in the hours immediately following a meal is lower than 7 (Van Soest, 1994). The values presented here point to the presence of saliva in the rumen fluid samples. The buffering activity of saliva did not allow the average sample pH value to drop below 7. The high standard deviation for the molar concentration of rumen fluid VFA is related to the fact that samples were contaminated with saliva to variable degrees. Our objective in

analysing rumen fluid was to determine the effects of Ca prop on the (acetate + butyrate) to propionate ratio. This ratio is not affected by contamination with saliva, since the dilution does not change the VFA proportions within a given sample. Our results show that the level of Ca prop fed increased the concentration of rumen propionate relative to acetate and butyrate concentrations at 2 h after feeding, but this effect was not sustained until 4 h after feeding. The addition of 300 g of Ca prop d^{-1} (150 g per meal) was enough to significantly decrease the molar ratio of (acetate + butyrate) to propionate in the rumen fluid at 2 h after feeding, even when this addition reduced DMI. This significant effect of Ca prop was observed regardless of the change in the pattern of intake, where animals on Ca prop would probably have eaten proportionally less of their total DMI during the first 2 h post-feeding if compared to control. The question remains as to whether there is a rate of inclusion of Ca prop in the diet of dairy cows that will not decrease DMI while still significantly altering the molar ratio of VFA in the rumen.

Plasma parameters

The lack of a significant difference between the control and the Ca prop diets in both insulin and glucose is probably due to the observed change in DMI and to the fact that the pancreas of lactating cows is less responsive to insulin secretagogues. Any effects of propionate as a secretagogue for insulin were overridden by the powerful effects of DMI on insulin secretion. Except for propionate, the ingestion of all potential insulin secretagogues decreased with the lower DMI observed when animals were fed the Ca prop diet. For ruminant animals, the release of insulin from pancreas after a meal is dependent on neural signals, gastrointestinal hormones, volatile fatty acids, amino acids and fat (Weekes, 1989). The study of any of these as a single factor is only possible when others are kept constant; since this is not the case in this experiment, the lack of a response in insulin secretion does not refute propionate as an insulin secretagogue. The maintenance of virtually equal levels of insulin in plasma between treatments is in part the result of an interplay between higher propionate intake coupled with decreased ingestion of all other secretagogues, comparing Ca prop diet to the control diet.

Lactation is a physiological state that alters pancreatic response to secretagogues. Arterial concentrations of insulin doubled in non-lactating cows during a 3 h infusion of propionate into a mesenteric vein, but were not statiscally different in lactating cows (Lomax et al., 1979), leading these authors to conclude that insulin secretion in response to an insulinotropic agent is diminished during lactation. Bigner et al., in 1996, further confirmed the fact that insulin secretion is decreased during lactation, showing an effect of metabolic acidosis on insulin response to glucose tolerance tests. Their results showed that plasma glucose concentrations were highest and plasma insulin concentrations were lowest after a glucose tolerance test (500 mg of glucose/kg of BW infused IV over 10 min) in non-lactating, non-pregnant mature Jersey cows undergoing metabolic acidosis due to a high anion diet. Although insulin responsiveness to insulin secretagogues is lower during lactation, there are two reasons for pursuing feed ingredients that lead to higher plasma insulin during lactation: (1) there is a certain degree of responsiveness (Bigner et al., 1996), which tends to increase as lactation progresses and (2) the appearance of studies showing insulin's ability to improve milk production (Léonard and Block, 1997; McGuire et al., 1995)

CONCLUSION

This work shows that Ca prop is a potential feed ingredient to increase protein percentage in milk, although the underlying mechanism is not clear. Further research is needed to determine the effects of Ca prop on milk, fat and protein yields without the confounding effects of a decreased DMI. The value of Ca prop as a feed ingredient to increase both protein percentage and yield is dependent on the answer to this question: is there an amount of Ca prop to be added in the diet of dairy cows that is low enough not to have negative effects on DMI and yet high enough to significantly stimulate the synthesis of milk protein in the mammary gland?

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Figure 1. Schematic representation of the design of each experimental period.

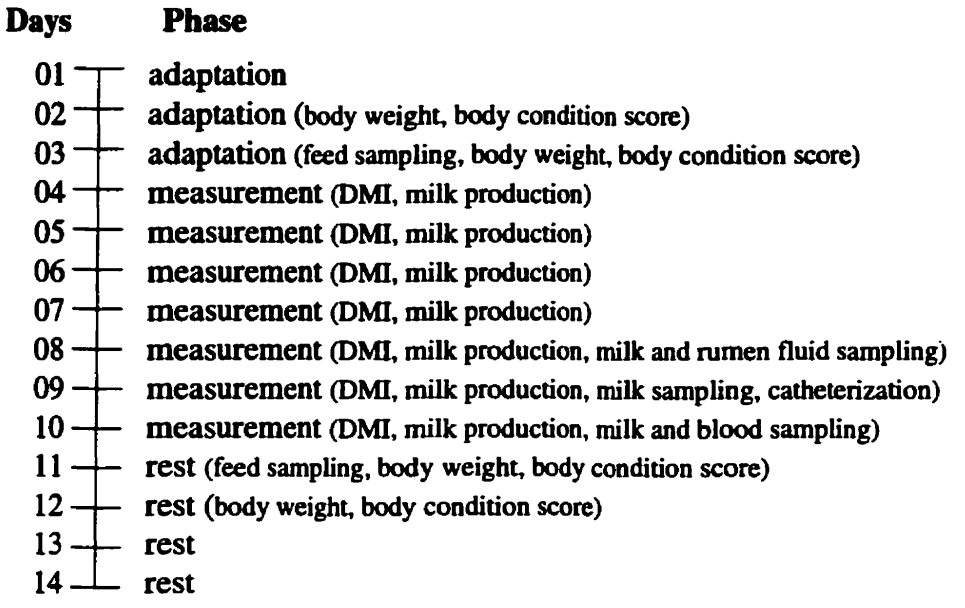

Figure 2. Effects of oral calcium propionate on dry matter intake and milk production of 12 post-peak Holstein cows (* denotes a significant difference; P<0.05).

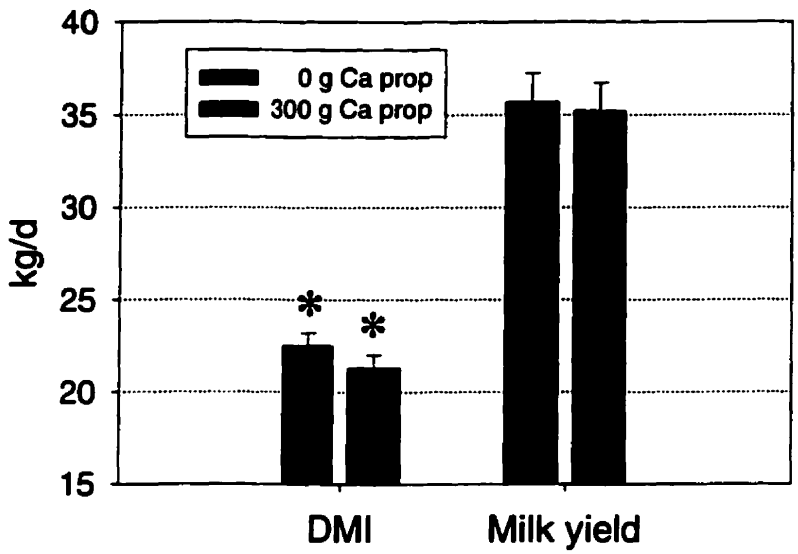

Figure 3. Effects of oral calcium propionate on milk protein percent of 12 post-peak Holstein cows (P<0.05).

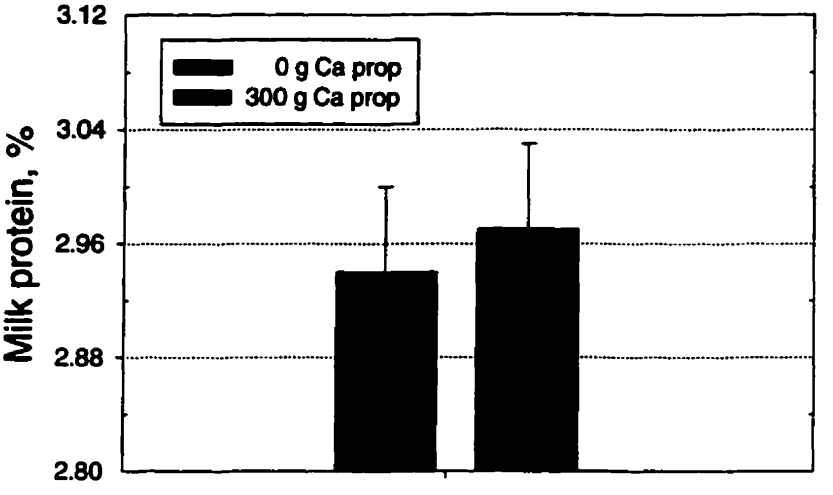

Figure 4. Ratio of acetate + butyrate to propionate ((A+B):P) in rumen fluid for each treatment, at 2 and 4 h after feeding, of 12 post-peak Holstein cows (* denotes a statistically significant difference; $P<0.05$).

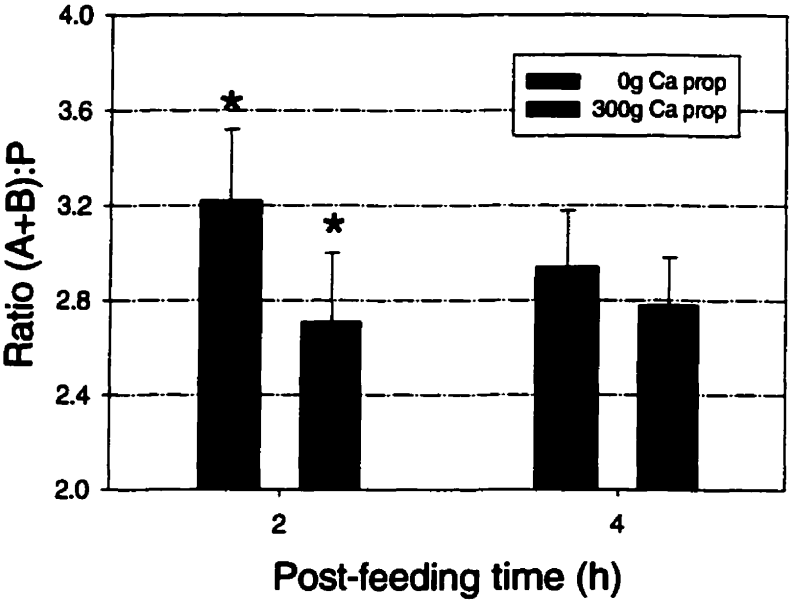

Figure 5. Plasma insulin concentration (pmol/L) of 12 post-peak Holstein cows. The difference between treatments for each hour was not significant (SE = 13.3 pmol/L; P>0.05).

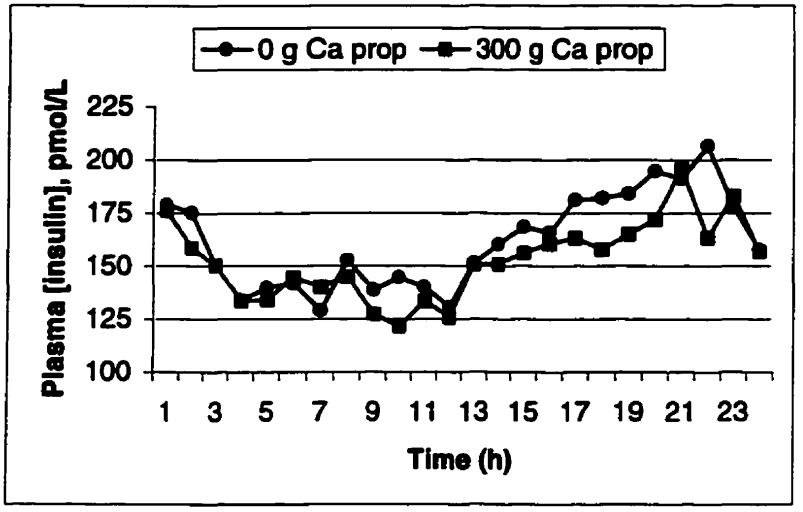

Table 1. Ration composition.

Component	Period (Replicate)					
	1(1)	2(1)	3(1)	1(2)	2(2)	3(2)
DM, %	43.9	45.0	45.5	43.2	46.9	46.1
CP, %	16.9	17.3	18.3	16.4	18.6	19.4
ADF, %	21.8	20.6	19.8	21.8	18.2	19.0
NDF, %	32.9	32.9	31.8	34.7	30.1	31.3
Ca, %	1.37	1.45	1.59	1.42	1.42	1.33
P, %	0.54	0.57	0.61	0.54	0.59	0.60
Mg, %	0.32	0.32	0.37	0.32	0.36	0.39
K, %	1.17	1.30	1.29	1.32	1.27	1.28
TDN, %	69.1	69.9	70.4	69.1	71.4	70.9
Calculated NEl, Mcal/kg	1.57	1.59	1.60	1.57	1.63	1.61

Table 2. Least square means for dry matter intake (DMI) and milk, protein and fat yields according to amounts of calcium propionate fed to 4 Holstein cows in early lactation (preliminary work).

Ca prop (g/d)	DMI (kg/d)	Milk yield (kg/d)	Protein yield (g/d)	Fat yield (g/d)
0	24.1[a]*	44.0[a]	1278[a]	1542[a]
200	24.0[a]	46.5[b]	1329[a]	1665[a]
350	22.6[b]	44.6[a,b]	1241[a]	1705[a]
500	22.1[b]	42.6[a]	1274[a]	1738[a]

* Different letters in the same column represent a statistically significant difference ($P<0.05$).

Table 3. Oral calcium propionate effects on fat content, fat production, protein production, lactose content and lactose production of 12 post-peak Holstein cows.

Trait*	0 g prop/d	300 g prop/d	SEM
Fat, %	3.16	3.09	0.17
Fat, kg/d	1.11	1.09	0.07
Protein, kg/d	1.04	1.04	0.05
Lactose, %	4.66	4.65	0.05
Lactose, kg/d	1.66	1.64	0.09

* No statistically significant differences were found between treatments for these variables ($P>0.05$).

Table 4. Overall average mM concentrations (SD) of VFA in rumen fluid[1] of 12 post-peak Holstein cows.

Time relative to feeding (h)[2]	Acetate	Propionate	Butyrate
2	118.7 (66.9)[3]	50.0 (24.8)	27.0 (11.9)
4	102.8 (68.4)	43.6 (28.9)	21.4 (11.2)

[1] Concentrations were averaged across treatments since there was no significant difference between the control and the calcium propionate diets.
[2] Refers to hours after morning feeding.
[3] The high standard deviation values are due to contamination of samples with saliva, probably masking any treatment effects.

CONNECTING STATEMENT

The following experiment was performed to bypass diet stimulation of insulin secretion through the intravenous infusion of the hormone, testing insulin effects on IGF-I secretion and production traits, as well as the interaction between insulin and somatotropin on these variables. The results are reported on the next two manuscripts.

CHAPTER 3

Manuscript 2

Effects of Insulin, Recombinant Bovine Somatotropin (rbST) and their Interaction on IGF-I Secretion and Milk Protein Production in Dairy Cows

By

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ABSTRACT

This trial was designed to test the effects of insulin, rbST and their interaction on milk protein and selected blood parameters in dairy cows. Eight Holstein cows (86 ± 10 DIM) were divided in 2 groups and used in 2 replicates of a Latin Square design with 4 animals, 4 periods and 4 treatments: (1) intravenous infusion of saline, (2) infusion of saline and subcutaneous administration of 40 mg of rbST per day, (3) intravenous infusion of 12 mg of insulin per day coupled with glucose infusion and (4) rbST administration combined with insulin and glucose infusion. The glucose infusion rate was adjusted to maintain normoglycemia. Each experimental period lasted 14 d: treatments were administered during the first 6 d and no treatment was administered during the following 8 d resting phase. The average daily amount of glucose infusion needed to avoid hypoglycemia was 2.8 kg/cow when only insulin was infused as opposed to 2.2 kg/cow when both insulin and rbST were administered, thus confirming the theory that rbST causes a peripheral resistance to insulin. Data from the last 3 d of infusion were analysed using the SAS system for mixed models. Percent protein of milk tended to be lower (2.79 vs 2.84%, $P=0.07$) and milk urea content was lower (14.8 vs 16.6 mg/dl, $P<0.01$) during rbST administration, regardless of insulin infusion. Insulin infusion increased percent protein (2.78 vs 2.85%, $P<0.01$), percent casein (2.36 vs 2.46%, $P<0.01$) and decreased milk urea content (17.1 vs 14.3 mg/dl, $P<0.01$) regardless of rbST administration. For milk yield, protein yield, casein yield, lactose percent and lactose yield, there was a significant interaction ($P<0.01$) between insulin and rbST administration. For example, casein yield averaged 1.17, 1.12, 1.20 and 1.28 kg/d for saline, insulin, rbST and insulin combined with rbST, respectively. Similarly, there was a significant interaction between insulin and rbST on plasma IGF-I levels, which were 122.5, 181.3, 342.3 and 492.2 ng/ml for saline, insulin, rbST and insulin combined with rbST, respectively. In conclusion, these results clearly demonstrated that insulin interacts with bST in early lactation to improve milk protein synthesis and yield in dairy cows. These effects are probably mediated through a combination of bST nutrient mobilisation, bST induced insulin peripheral resistance and bST/insulin synergism on IGF-I secretion and on mammary epithelial tissue. (Key words: insulin clamp, bST, milk protein, IGF-I, dairy cows)

INTRODUCTION

From an economic perspective, dairy producers and processors rely on production of individual milk components rather than overall milk yield, with an increasing emphasis on milk protein. The expression of the demand for milk protein is twofold: pressure from the consumer, who demands healthier food, and its impact on the producer, who sees increase revenue for higher protein production. Furthermore, protein is the single most important component in milk for manufacturing purposes (Hettinga, 1989). Many researchers have tried to improve milk protein production by enhancing AA supply (Clark, 1975; Rulquin et al., 1995). However, milk protein yield is related to a greater extent to energy intake than to protein intake (DePeters and Cant, 1992; Spörndly, 1989; Sutton, 1989). Thus, milk protein synthesis by the mammary gland is not solely the assembly of AA coming straightforward from dietary protein. It becomes then important to understand how milk protein synthesis is controlled by the dairy cow. Research by two independent laboratories, working with insulin infusion, has shown that insulin is a regulator of milk protein synthesis in lactating dairy cows (Léonard and Block, 1997; Griinari et al., 1997b; Mackle et al., 1999 and McGuire et al., 1995b). Additionally, both endogenous bovine somatotropin (bST) and exogenous recombinant bovine somatotropin (rbST) are involved in regulating insulin effects in dairy cows. For example, the actions of rbST in lactating cows rely in part on the modification of insulin effects on peripheral tissues (Burton et al., 1994). The mechanism of rbST actions on the yield of milk and its components is related to an increase in plasma IGF-I concentration (Peel and Bauman, 1987). However, insulin is also able to increase plasma IGF-I levels (McGuire et al., 1995a). Therefore, in order to better understand the effects of insulin and rbST on production of milk and its components, it is crucial to study the interaction between both hormones. The objective of this trial was to evaluate the effects of exogenous insulin, rbST and their interaction on both milk yield and milk protein percentage, yield and composition in lactating dairy cows.

MATERIALS AND METHODS

Animals and treatments

Eight Holstein cows averaging 86 ± 10 DIM were used in 2 replicates of a Latin Square design with 4 cows, 4 periods and 4 treatments. Each experimental period lasted 14 d: treatments were administered during the first 6 d and no treatment was administered during the following 8 d resting phase, as shown in Figure 1. The treatments were (1) IV infusion of saline, (2) IV infusion of saline and subcutaneous administration of 40 mg of rbST (American Cyanamid Company, Princeton, NJ) per day, (3) IV infusion of 12 mg of insulin (# I 5500, Sigma-Aldrich Canada Ltd, Oakville, Ontario) per day coupled with a variable rate of glucose infusion and (4) subcutaneous administration of 40 mg of rbST per day combined with IV insulin and glucose infusion. The solutions used for IV infusion are described in Table 1. Recombinant bST was injected daily at 23:00 h for 6 consecutive days during each period, starting on day zero. The guidelines of the Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, 1993) were observed for all procedures involving the animals.

Management and measurements

The cows were housed in an artificially lighted and ventilated tie-stall barn with separated mangers that allowed the measurement of individual feed intake. All animals received the same diet in the form of a total mixed ration (TMR) with a 40:60 ratio of haylage (alfalfa and timothy) + corn silage to grains, formulated according to the requirements of the National Research Council (1989) and fed at around 110 % of their ad libitum intake, in 2 meals per day. During the infusion phase, feed offered and feed refused were weighed daily. Samples of the TMR were collected on d -1, 0, 7 and 8 of each period, immediately before and after the infusion; these samples were analysed using traditional wet chemistry procedures for acid detergent fiber (Van Soest, 1973) and

neutral detergent fiber (Goering and Van Soest, 1970). Crude protein, ether extract, calcium, phosphorus and ash were determined according to A.O.A.C (1985) procedures. Dry matter was determined using a forced draft equipment at 100°C (Koster Crop Tester, Cleveland, Ohio). Table 2 shows the average composition of the TMR during the experimental periods. Milking was performed 3 times daily (08h00, 16h00 and 24h00).

On d 0, an indwelling catheter (IV Intracath – catheter-needle unit, vialon, gauge 16, 8 in long, needle 14, Becton-Dickinson, cat # 3831621) was installed in the left jugular vein and was maintained for the duration of treatments throughout the 6 d of the infusion phase. The catheter was connected to an extension that crossed from the barn to the infusion room, passing through the barn wall. In the case of animals receiving saline, the extension led to a bottle containing saline solution. When animals were receiving insulin and glucose, the extension was connected to 3 shorter extensions: 2 led to bottles containing the glucose solution and 1 led to the insulin solution. The glucose and insulin solutions were kept constantly refrigerated at 4 °C. The pump used for the insulin and saline infusions (Gilson peristaltic pump, miniplus 2, Gilson France SA, Villiers le Bel, France) was kept at a constant flow rate (2 ml/min). There were 2 other pumps (Masterflex peristaltic pump, Cole Palmer Instruments Co., Chicago, IL) which were used for the glucose infusion; each pump served 1 of the 2 cows that were receiving the insulin + glucose infusion within the same period. The flow rate of the glucose infusion was adjusted individually for each cow every two hours, according to blood glucose values read in the barn with a glucometer (Accu-Chek Advantage, Boehringer Mannheim, Hoffmann-La Roche Ltd., Quebec, Canada). The objective of the glucose flow rate adjustments was to maintain glycemia within -10% and +20% of baseline values. Therefore, a narrower variation was allowed for blood glucose levels below baseline, since the priority was to avoid hypoglycemia. The baseline values were an average of 4 blood glucose measurements taken on d 0 at 19:00, 21:00, 23:00 and 24:00 h. The infusion started on d 1, at 01:00 h. Blood glucose level of cows receiving insulin and glucose infusion was measured every 20 min, until values fell within the expected range, which occurred on average during the first 3 initial h. After that, blood glucose

was measured every 2 h during the 6 d of infusion, and glucose infusion rate was adjusted accordingly. On d 5, the animals were fitted with an indwelling catheter in the right jugular vein to be used for the hourly blood sampling. On the last day of the infusion phase (d 6), blood samples were collected for the determination of plasma levels of insulin, glucose, rbST and IGF-I; hourly samples were collected from 01:00 h to 24:00 h. Each blood sample was collected into 2 different tubes: (1) a tube with sodium fluoride and potassium oxalate for glucose analysis and (2) a tube with sodium heparin for insulin, rbST and IGF-I analysis. The samples were immediately put on ice and centrifuged (6,000 x g for 20 minutes) within 15 min. Plasma was then aliquoted and frozen.

Frozen plasma samples were analysed for glucose content by automated spectrophotometry (Abbott VP Super System, Abbott Laboratories, Mississauga, Canada), using a commercial kit (Sigma Diagnostics, St. Louis, Mo, USA); inter- and intraassay coefficients of variation were 1.21 and 0.89 %, respectively. Insulin analysis was done by a RIA procedure using a commercial kit (KTSP-11002, Immunocorp, Montreal, Canada); inter- and intraassay coefficients of variation were 7.96 and 4.12 %, respectively. Levels of rbST were measured as described by Petitclerc et al. (1987); inter- and intraassay CV were 4.63 and 3.22 %, respectively. A RIA method was used for the determination of IGF-I levels as described elsewhere (Abribat et al., 1993); inter- and intraassay CV were 5.10 and 2.68%, respectively.

Within each period, milk yield and milk samples for component analyses were taken during d 4, 5 and 6: d 1, 2 and 3 were considered an adaptation phase (animals received treatments but the production data were not included in the statistical analysis). Double milk samples were taken at each milking. One set of milk samples was sent to the Programme d'Analyse des Troupeaux Laitiers du Québec (PATLQ) where protein, lactose and urea contents were determined with an infrared analyser. The second set was used to determine casein content of milk as the difference between total protein and whey protein content of milk. Total nitrogen content of milk and the non-casein nitrogen

content of acid whey, after isoelectric precipitation of caseins, were determined using a Leco FP428 analyser (Leco Corp., St-Joseph, MI 45085, USA).

Statistical analyses

Data were analysed using the proc MIXED of SAS system for mixed models (SAS Institute, 1996). Replicate, period within replicate, rbST, insulin, day and hour were considered fixed effects. The effect of cow was included in the random statement. Hour was included in the repeated measures statement. The proc MIXED was used because it allows modelling the covariance structure, which is especially important for the analysis of repeated measures data (Littell et al., 1998). Different covariance structures were tested for plasma hormones and glucose; the auto regressive covariance order 1 was the best and was used for the analyses of these variables. All other traits were analysed using the compound symmetry covariance structure. Production parameters were analysed by model [1]; plasma hormones and glucose were analysed by model [2].

$$Y_{ijklmn} = \mu + Rep_i + Per_j(Rep_i) + rbST_k + Ins_l + rbST_k*Ins_l + C_m(Rep_i) + D_n + e_{ijklmn} \quad [1]$$

$$Y_{ijklmn} = \mu + Rep_i + Per_j(Rep_i) + rbST_k + Ins_l + rbST_k*Ins_l + C_m(Rep_i) + H_n(Per_j*rbST_k*Ins_l) + e_{ijklmn} \quad [2]$$

In these models, Y is the measured parameter, μ is the overall mean, Rep is the replicate effect, Per is the period effect, rbST is the effect of the subcutaneous injection of 40 mg of rbST per day, Ins is the effect of the infusion of 12 mg of insulin per day, C is the cow effect, D is the day effect, and H is the hour effect.

All parameters measured, with the exception of those from blood origin, were assumed to have a homogeneous variance, and the variance estimates are shown in Table 3. The variance across treatments for plasma glucose, insulin, bST and IGF-I levels was not homogeneous. The group statement in SAS (SAS Institute, 1996) was used to adjust the

analysis for the heterogeneity of variance. The grouping that best described this heterogeneity in variance were by insulin infusion levels for the statistical analysis of plasma insulin and IGF-I and by the interaction rbST*insulin for plasma glucose and bST (Table 4).

RESULTS

All animals completed the study in excellent health and returned to the regular management at Macdonald Campus Dairy Complex of McGill University.

Rate of glucose infusion

The average daily amount of glucose infusion needed to avoid hypoglycemia throughout the last 3 d of the clamp was 2.8 kg when cows received only insulin as opposed to 2.2 kg when cows received insulin and rbST (Table 5), confirming that rbST causes a peripheral resistance to insulin. The rate of glucose infusion needed to maintain glycemia increased throughout the clamp (Figure 2), especially for cows not receiving rbST. The difference between treatments regarding the amount of glucose needed for normoglycemia maintenance was most marked on d 5 and 6 of the clamp (Figure 2).

Production traits

Table 6 shows the least square means (LSM) for milk protein content (%), casein content (%), casein as a percentage of total protein (%), and urea content (mg/dl) of milk. For these traits, the interaction between insulin and rbST was not statistically significant ($P>0.10$); therefore, only the main effects of these hormones are shown. Protein content tended to be lower ($P=0.07$), casein content was lower ($P<0.01$), casein as a percentage of total protein was not affected ($P>0.10$), and milk urea content was lower ($P<0.01$) during

rbST administration. Insulin infusion increased protein content (P<0.01), casein content (P<0.01) and casein as a percentage of total protein (P=0.01), and decreased milk urea content (P<0.01). For milk yield, protein yield, casein yield, lactose content and lactose yield, there was a significant interaction between insulin and rbST administration (P<0.01). Figure 3 shows the effects of insulin infusion within the two levels of rbST on casein yield. The graphic representation of the interaction between insulin and rbST treatments on milk yield, lactose yield, and protein yield presented the same pattern as for casein yield (Figure 3). The infusion of 12 mg of insulin per day in the absence of rbST administration causes a decrease in yield of these traits, but the same infusion of insulin in the presence of the administration of 40 mg of rbST per day leads to an increase in their yields. Table 7 shows the LSM for milk yield, protein yield and casein yield for all the comparisons within the interaction that were statistically significant. Average SCC throughout the experiment was 94 ± 156 cells*10^3/ml, and it was not statistically analysed because it was not normally distributed.

Plasma parameters

There was a 96 % correlation between the two methods of glucose determination used in this trial, the glucometer and the more traditional automated spectrophotometry. Normoglycemia was maintained across treatments and there was no effect of insulin + glucose infusion, rbST administration or the interaction between both treatments on plasma glucose levels on d 6 (Figure 4). Levels of plasma glucose averaged 57.1 ± 0.7 mg/dl when animals received saline infusion and 58.0 ± 1.2 mg/dl when animals received insulin + glucose infusion. Insulin infusion showed a significant effect on plasma insulin levels (P<0.01), being 1.08 ± 0.06 ng/ml when animals received saline infusion and 1.72 ± 0.07 ng/ml when animals received insulin + glucose infusion (Table 6 and Figure 5). Recombinant bST administration significantly increased plasma levels of bST (P<0.01), which averaged 2.6 ± 0.1 and 15.7 ± 3.2 ng/ml for saline and rbST administration, respectively (Table 6 and Figure 6). Plasma levels of IGF-I averaged 122.5 ± 43.5, 181.3 ± 28.8, 342.3 ± 43.5 and 492.2 ± 28.8 ng/ml for saline, insulin, rbST and insulin + rbST

treatments, respectively (Table 7 and Figure 7). The effect of the interaction between insulin and rbST on IGF-I levels was statistically significant ($P<0.05$) (Figure 8). Table 7 shows the LSM for plasma IGF-I levels for all the comparisons where treatment differences were statistically different from zero.

DISCUSSION

The number of studies using the hyperinsulinemic-euglycemic clamp in dairy cows has increased over the last decade. This technique has been successful as a tool to study the effects of insulin administration while maintaining normoglycemia. Many factors interact to determine the rate of glucose infusion needed for the maintenance of normoglycemia, such as DIM, basal levels of plasma insulin and levels of plasma insulin attained during its administration. Léonard and Block (1997) infused a fixed amount of 1.2 kg of glucose/d in cows throughout the clamp period before peak lactation and the animals became hypoglycemic. The average daily amount of glucose infusion needed to avoid hypoglycemia in our cows was in the range of that used for cows 184 DIM, whose normoglycemia was maintained with 2.5 kg of glucose/d (Griinari et al., 1997a and b). However, it was lower than the 3.3 kg of glucose/d reported by Mackle et al. (1999); possible reasons for this are the larger increase in insulin levels obtained and the fact that their cows were 220 ± 11 DIM. In terms of glucose rate of infusion, McGuire et al. (1995b) found that it became relatively constant from d 2 through the end of the clamp period (d 4). Although the clamp period in our trial was longer, it was not observed that glucose requirements for normoglycemia stabilized with time, especially when cows did not receive rbST. Indeed, throughout the 6 d of the clamp, the amount of glucose infused had to be increased. This is in accordance with the infusion rate curve presented by Mackle et al. (1999). These results confirm that, in the establishment of a hyperinsulinemic-euglycemic clamp in dairy cows, it continues to be essential to monitor blood glucose levels and adjust the rate of glucose infusion accordingly. Furthermore, these results provide strong evidence that rbST injection induced a resistance to insulin effect on glucose utilisation by peripheral tissues.

Insulin infusion significantly increased the concentration of protein in milk, a response previously observed (McGuire et al., 1995b; Léonard and Block, 1997). However, all effects of insulin reported in the literature have been consistently confounded with DIM. All previous studies with the hyperinsulinemic-euglycemic clamp in cows that succeeded in maintaining normoglycemia (McGuire et al., 1995a,b; Griinari et al., 1997a,b; Mackle et al., 1999) were designed so that the control period was a baseline period before the clamp started. Hence, when cows received insulin infusion, they were also later in lactation if compared to the baseline period. It is known that, as lactation progresses, the percentages of protein and fat in milk tend to increase and the yield of milk and components tends to decrease. The same is true for plasma levels of insulin, bST and IGF-I, which tend to increase (Vasilatos and Wangsness, 1981), decrease (Vasilatos and Wangsness, 1981) and increase (Gallo and Block, 1990), respectively, as lactation progresses. An important feature of the results presented in this study is that they were obtained using a Latin square design, so they are adjusted for the effect of time. The effect of insulin on increasing milk protein content is confirmed. This effect is even more pronounced when we consider casein content, since insulin significantly reduced NPN in the form of urea, as seen before (Mackle et al, 1999; Léonard and Block, 1997; Griinari et al., 1997b). We also observed that rbST treatment significantly reduced casein and urea contents in milk, and caused a marginal decrease in total protein content. This is in contrast to rbST effects reported before (for review see Burton, 1994) and might be related to the short timeframe of rbST administration used in this study and to the effects observed on DMI, discussed elsewhere (Molento et al., in preparation).

The 62.8 % increase in insulin levels observed in this study due to insulin and glucose infusion might represent a more physiological variation for dairy cows than the increases obtained with the clamp in other studies. These increases in plasma insulin levels vary from 122 % (Léonard and Block, 1997) to more than 500 % (McGuire et al., 1995b). Even though the increase in plasma insulin reported here was of a smaller magnitude, the increase in protein and casein contents, and the decrease in milk urea content were

significant and similar to those changes observed previously. As well, the increase in plasma insulin concentration attained was able to significantly alter the responses to rbST in terms of milk, lactose, protein and casein yield, and lactose content. The milk, lactose and protein yield, as well as casein yield responses showed great similarity to the interaction between insulin and bST on circulating IGF-I levels. Lactose yield and consequently milk yield are not directly related to insulin in the mammary gland, since lactose synthesis by the mammocytes is not regulated by insulin (Hove, 1978). However, our results show that insulin is able to modify the response of these variables to rbST administration. Insulin infusion led to a greater response in the yield of milk, lactose, protein and casein when rbST was administered. In other words, this illustrates the decreased ability of bST to increase milk, lactose, protein and casein yield when insulin levels are lower, which in turn might be related to the decreased secretion of IGF-I, referred to as the uncoupling of the bST-IGF-I axis (Léonard and Block, 1997; Burton et al., 1994). Because there was no difference in energy balance between treatments, it seems that plasma levels of insulin are involved in the uncoupling of this hormonal axis regardless of the energy balance itself. However, longer-term trials are needed before a final conclusion is made in this regard.

The magnitude of the increases in both plasma bST and IGF-I levels due to rbST administration is within the range reported in the literature (Schams et al., 1989). As reviewed by McBride et al. (1988) and supported by more recent research (Prosser et al, 1989; McGuire et al., 1995a), rbST significantly increases plasma levels of IGF-I; the same effect was observed in this study. In terms of insulin infusion, IGF-I has also responded with a significant increase as observed before, when plasma bST levels were either low in the order of 4.5 ng/ml (McGuire et al., 1995a) or higher due to simultaneous administration of rbST (Léonard and Block, 1997). However, it was quite interesting to observe a similar interaction between insulin and rbST on IGF-I levels and also on milk yield, milk protein yield and milk casein yield. It is thought that IGF-I is related to protein synthesis in the mammary gland (Zhao et al, 1992), and the similarity between

IGF-I levels and milk/protein yield responses to treatments is another indication of a strong role of IGF-I on milk protein synthesis.

The mechanism of action of the insulin clamp on milk protein synthesis is not fully understood. Although a direct effect of insulin on milk yield has not been reported, it is likely that insulin participates in bST stimulation of milk production and is probably mediated indirectly by IGF-I. Indeed, in cows subjected to a hyperinsulinemic-euglycemic clamp, there is an increase in IGF-I (McGuire et al., 1995a; Léonard and Block, 1997) and a decrease in IGF-II and insulin-like growth factor binding protein 2 (McGuire et al., 1995a). The bST to insulin ratio might be a better indicator to predict IGF-I synthesis by the liver than bST levels alone. This ratio could explain variations in the ability of bST to stimulate liver IGF-I production under different nutritional and physiological conditions. Therefore, increases in milk proteins due to insulin might be (a) direct, via the effects of insulin either on the stimulation of mammary epithelial cell proliferation or on AA transport systems on mammary cells; (b) indirect, via increases in IGF-I combined with changes in insulin-like growth factor binding protein levels or also via changes in bST receptors in the liver affecting GH stimulation of IGF-I release (Léonard et al., unpublished results) or (c) a combination of both.

CONCLUSION

In conclusion, these results clearly demonstrated that insulin interacts with bST in early lactation to improve milk yield and milk protein synthesis in dairy cows. These effects are probably mediated through a combination of effects of bST on nutrient mobilisation, bST induced insulin peripheral resistance and bST / insulin synergism on IGF-I secretion and on the metabolic activity of the mammary tissue. The confirmation of insulin's ability to increase milk protein content, even with the modest increase in insulin plasma level observed here, warrants further studies with the goal of manipulating the diet of dairy animals so as to increase pancreatic stimulation that leads to increased endogenous insulin release. Such feeding strategies to enhance insulin secretion might be especially

relevant in cows with increase plasma bST concentrations.

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Figure 1. Schematic representation of the experimental design.

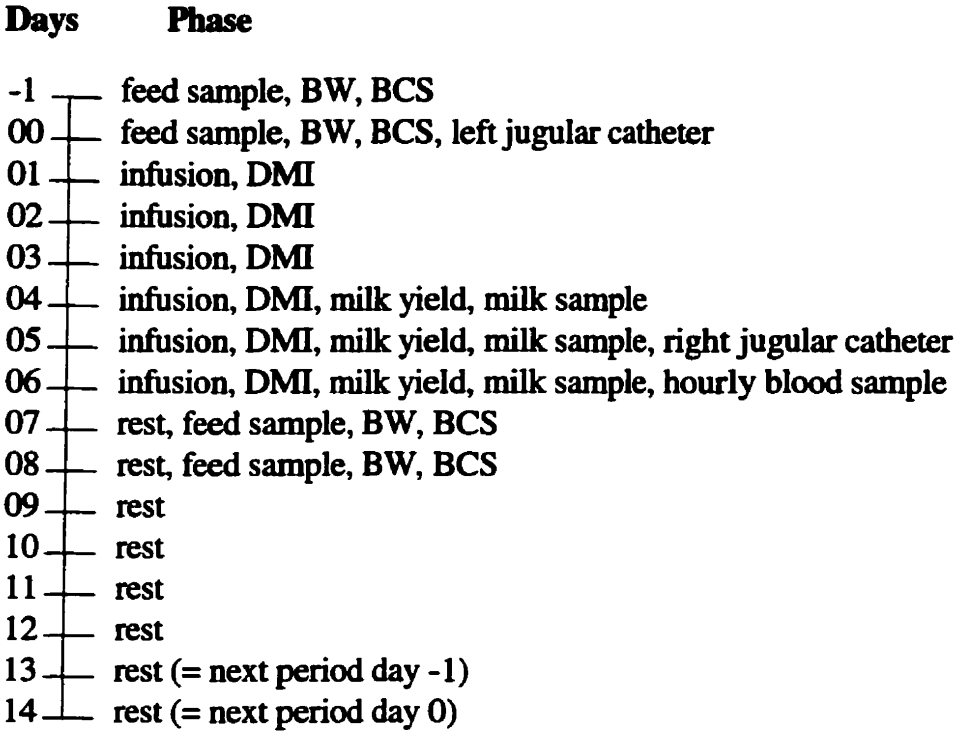

Figure 2. Glucose flow rate given during the insulinemic-euglycemic clamp: filled circles represent average values for insulin + glucose infusion and open circles represent average values for insulin + glucose infusion coupled with recombinant bovine somatotropin administration.

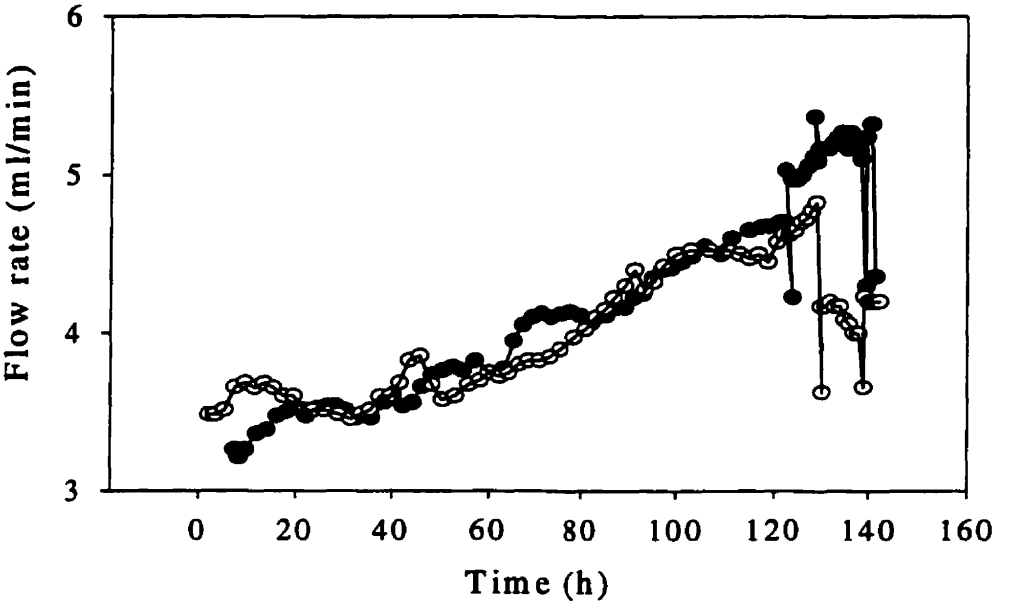

Figure 3. The effects of insulin + glucose infusion on casein yield within two levels of recombinant bovine somatotropin (rbST). The interaction between treatments was significant; refer to Table 7 for details on the significant comparisons within the interaction.

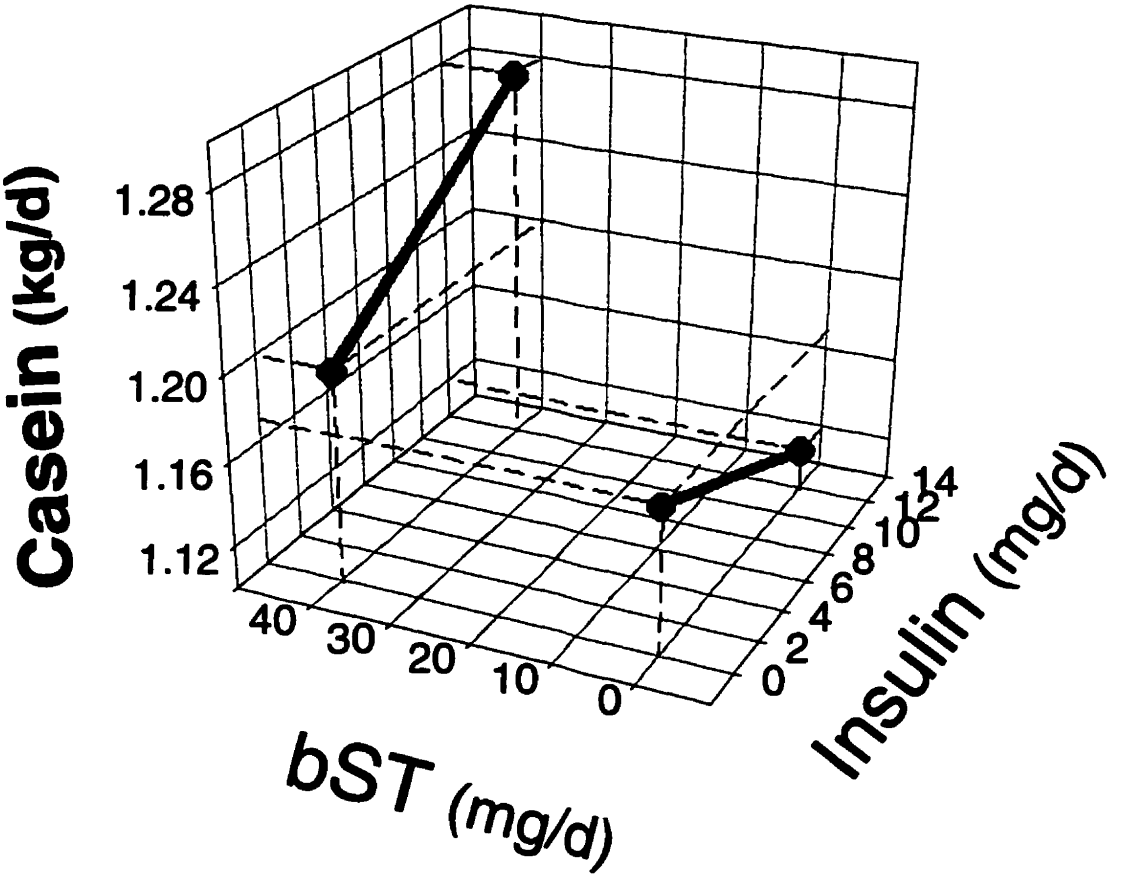

Figure 4. Average plasma glucose levels for saline (open square), recombinant bovine somatotropin (rbST) (filled square), insulin + glucose (open triangle) and insulin + glucose coupled with rbST (filled triangle) treatments; plasma samples were taken on day 6 of the infusion period.

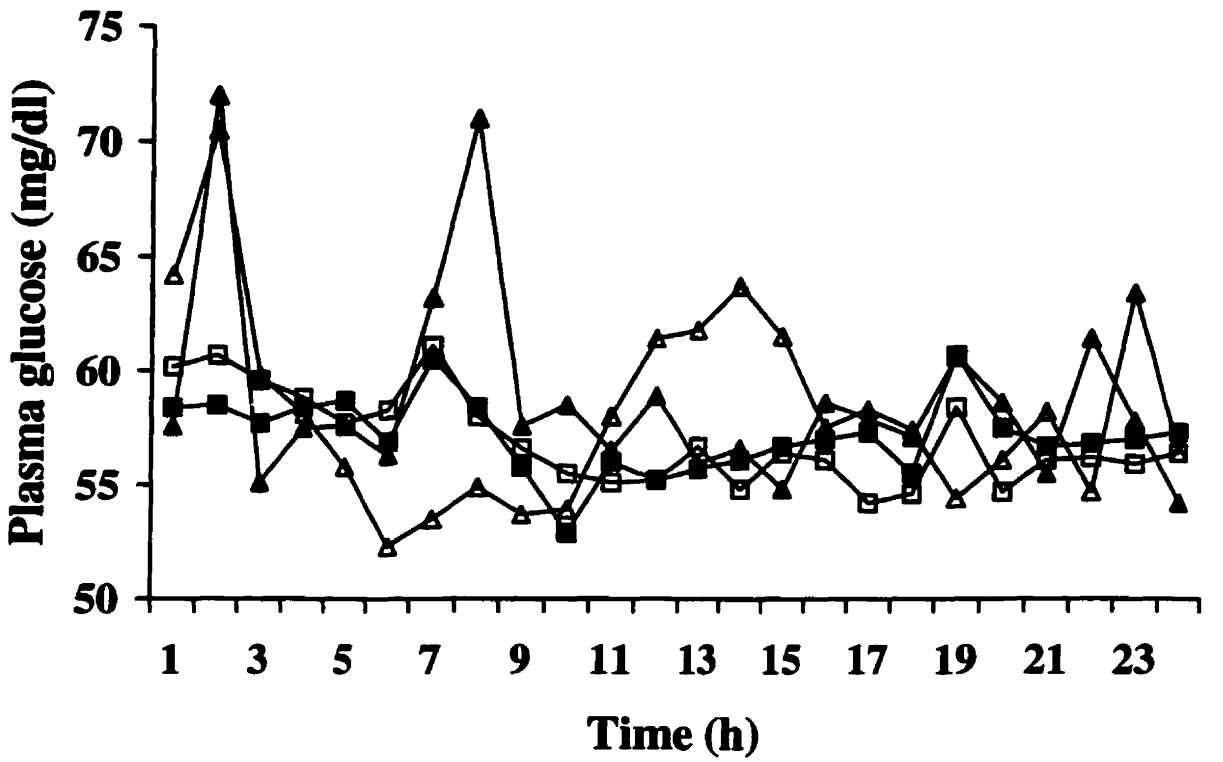

Figure 5. Average plasma insulin levels for saline (open square), recombinant bovine somatotropin (rbST) (filled square), insulin + glucose (open triangle) and insulin + glucose coupled with rbST (filled triangle) treatments; plasma samples were taken on day 6 of the infusion period.

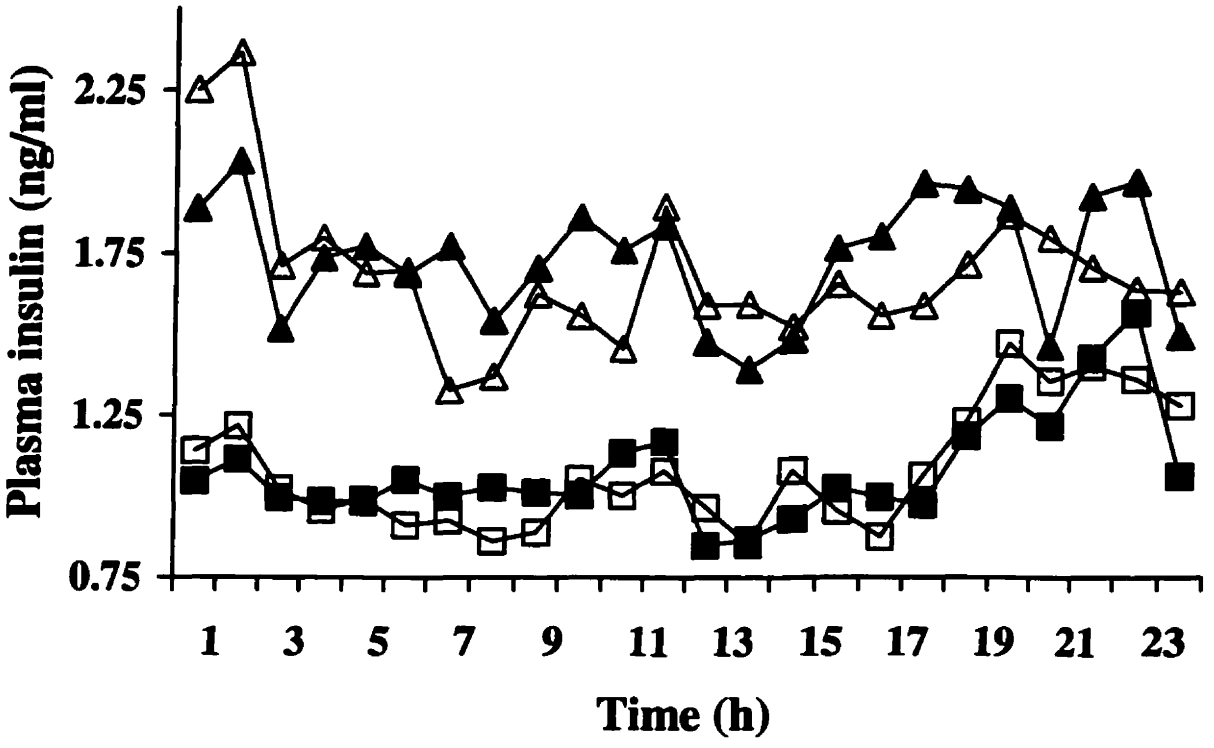

Figure 6. Average plasma bST levels for saline (open square), recombinant bovine somatotropin (rbST) (filled square), insulin + glucose (open triangle) and insulin + glucose coupled with rbST (filled triangle) treatments; plasma samples were taken on day 6 of the infusion period.

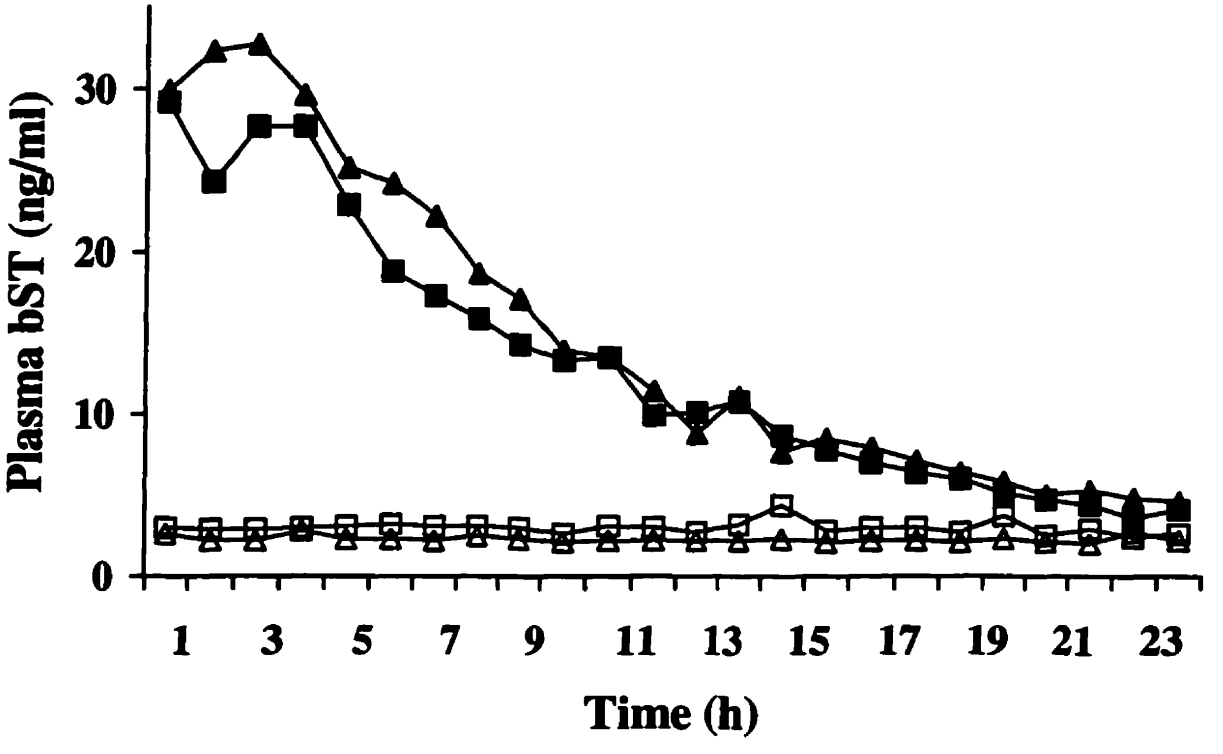

Figure 7. Average plasma IGF-I levels for saline (open square), recombinant bovine somatotropin (rbST) (filled square), insulin + glucose (open triangle) and insulin + glucose coupled with rbST (filled triangle) treatments; plasma samples were taken on day 6 of the infusion period.

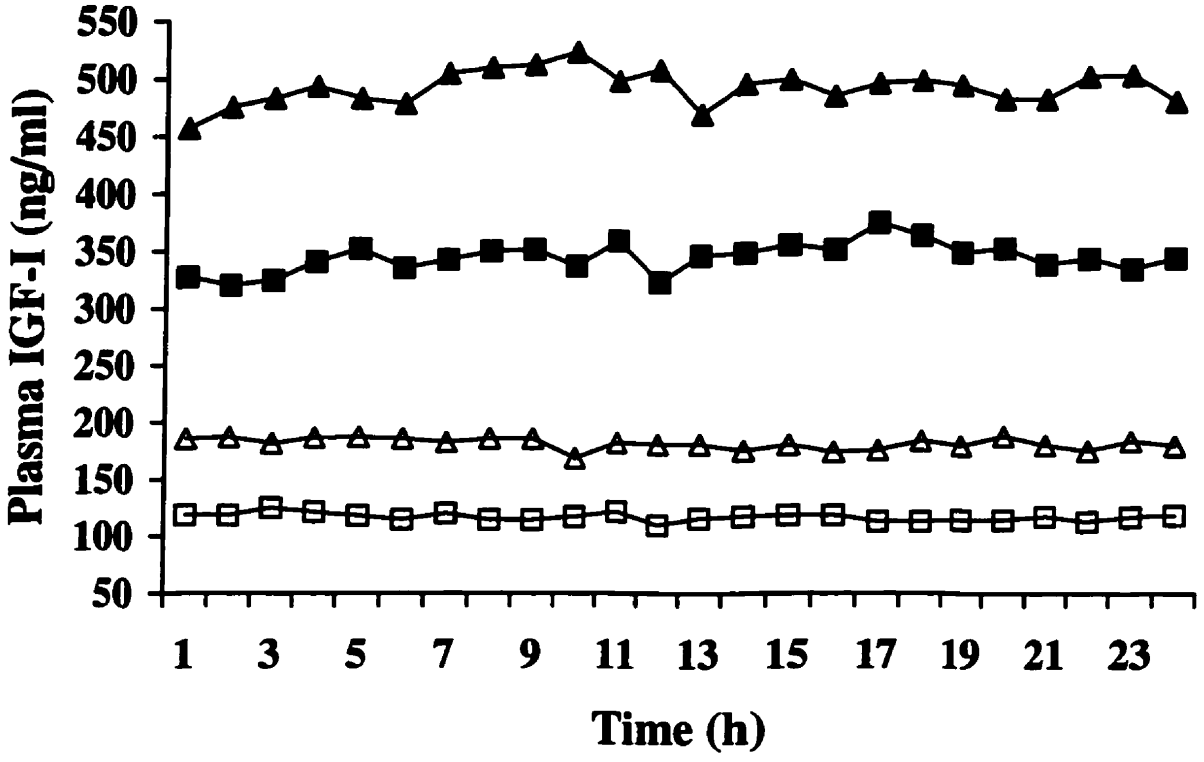

Figure 8. The effects of insulin + glucose infusion on IGF-I plasma concentrations within two different levels of recombinant bovine somatotropin (rbST). The interaction between treatments was significant; refer to Table 7 for details on the significance of comparisons within the interaction.

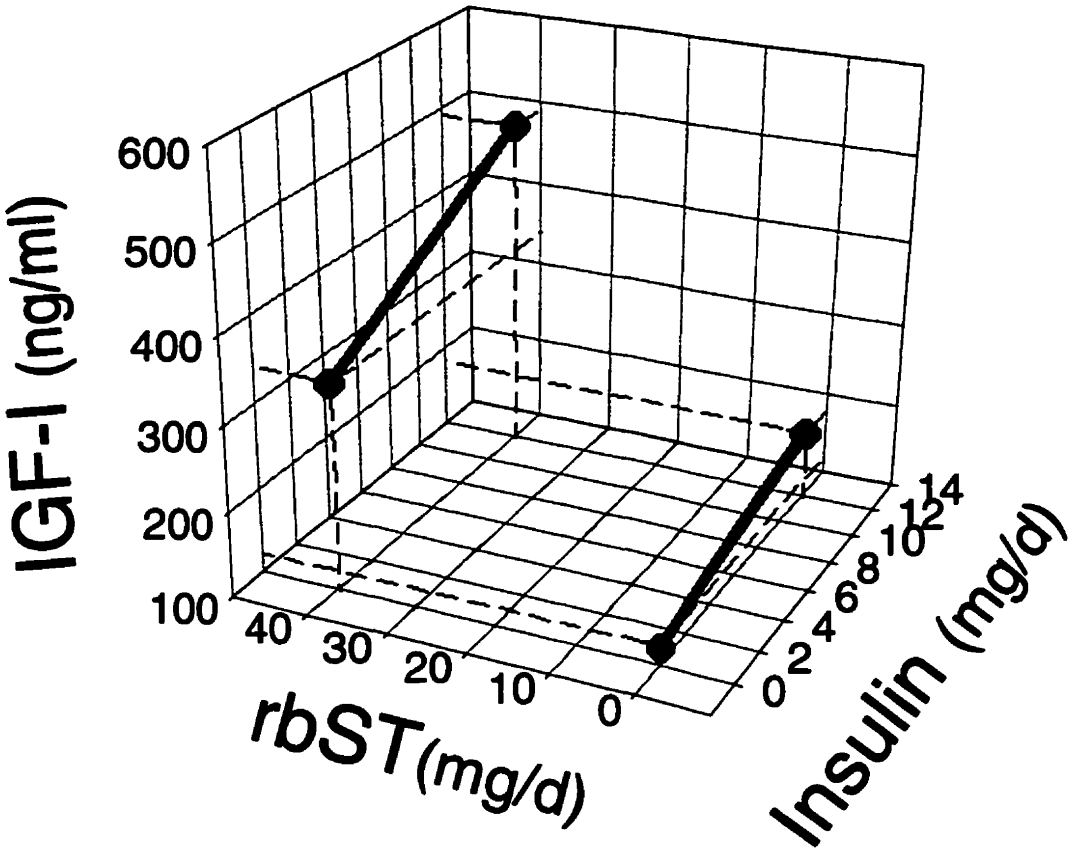

Table 1. Description of the solutions used for intravenous infusion.

Solution	Content	Concentration
Saline	NaCl	9 g/l
Glucose	NaCl	9 g/l
	Dextrose	500 g/l
Insulin	NaCl	9 g/l
	Dextrose	500 g/l
	Bovine insulin	4.17 mg/l

Table 2. Ration composition.

Component[1]	Experimental period (replicate)							
	1(1)	2(1)	3(1)	4(1)	1(2)	2(2)	3(2)	4(2)
DM, %	56.1	59.3	54.8	52.7	53.5	54.2	50.4	51.9
CP, %	17.9	18.3	16.2	17.8	17.1	18.6	18.1	18.7
ADF, %	16.5	12.6	16.5	19.3	16.4	15.8	19.1	17.9
NDF, %	28.3	20.4	28.6	32.7	27.7	26.5	30.5	27.3
Ca, %	1.01	1.18	0.87	1.00	1.07	1.03	1.08	1.24
P, %	0.51	0.54	0.48	0.53	0.49	0.50	0.48	0.53
Mg, %	0.32	0.34	0.31	0.36	0.32	0.34	0.32	0.31
K, %	1.04	1.03	0.99	1.13	1.27	1.17	1.39	1.51

[1]All component percentages were analysed from samples of the total mixed ration offered. NEl values were not calculated.

Table 3. Variance estimate for milk yield, protein yield, protein percent, casein yield, casein percent and milk urea.

Parameter	Cow variance	CS variance[1]	Residual variance
Milk yield, kg/d	24.98	3.276	16.119
Protein yield, g/d	0.022	-0.001	0.018
Protein, %	0.023	0.000	0.011
Casein yield, g/milking	0.002	0.000	0.003
Casein, %	0.034	0.000	0.029
Urea, mg/dl	2.557	0.183	1.747

[1]The compound symmetry covariance structure CS was used.

Table 4. Variance estimates for glucose, insulin, bST and IGF-I, according to the grouping that best described the heterogeneity of the variance[1].

Grouping parameter	Glucose			Insulin			bST			IGF-I		
	Cow[2]	Var.[3]	AR[4]	Cow	Var.	AR	Cow	Var.	AR	Cow	Var.	AR
Insulin, 0 mg/d	-	-	-	711.2	2687	0.378	-	-	-	6586	11442	0.97
Insulin, 12 mg/d	-	-	-	711.2	7693	0.369	-	-	-	6586	961.7	0.12
S[5]	2.69	14.9	0.467	-	-	-	0.123	1.246	0.056	-	-	-
I[5]	2.69	145.9	0.526	-	-	-	0.123	0.377	0.131	-	-	-
B[5]	2.69	14.6	0.461	-	-	-	0.123	202.7	0.952	-	-	-
I+B[5]	2.69	160.7	0.291	-	-	-	0.123	326.8	0.944	-	-	-

[1] The auto-regressive covariance structure AR(1) was used.
[2] Variance due to the effect of cow.
[3] Variance within grouping.
[4] Autoregressive covariance.
[5] Grouping codes: S = saline infusion, I = insulin and glucose infusion, B = bST administration, B + I = bST administration combined with insulin and glucose infusion.

Table 5. Total amount of glucose infused per 24 h (average of last 3 days of infusion).

Cow number	Period (replicate)	Treatment	g of glucose / 24 hrs
1113	2 (1)	I	2946
1113	3 (1)	I + bST	2745
1153	4 (1)	I	1979
1153	1 (1)	I + bST	2942
1213	3 (1)	I	3111
1213	4 (1)	I + bST	1094
1268	1 (1)	I	3794
1268	2 (1)	I + bST	2067
1072	1 (2)	I	3273
1072	2 (2)	I + bST	1888
1177	4 (2)	I	3155
1177	3 (2)	I + bST	3247
1245	3 (2)	I	1964
1245	1 (2)	I + bST	1832
1250	2 (2)	I	2038
1250	4 (2)	I + bST	1804
Average		I	2782
Average		I + bST	2202

Table 6. Least square means for milk protein content, casein content, casein as a percentage of total protein content (CPTP), milk urea content, plasma insulin and plasma bovine somatotropin (bST).

Trait	BST			Insulin		
	0 mg/d (A)	40 mg/d (B)	Pdiff [1]	0 mg/d (C)	12 mg/d (D)	Pdiff [1] 1.1.1.
Protein, %	2.84 (0.05)[2]	2.79 (0.05)	P=0.07	2.78 (0.05)	2.85 (0.05)	P<0.01
Casein, %	2.45 (0.07)	2.37 (0.07)	P<0.01	2.36 (0.07)	2.46 (0.05)	P<0.01
CPTP, %	81.1 (0.6)	80.8 (0.6)	P=0.27	80.7 (0.6)	81.2 (0.6)	P=0.01
Urea, mg/dl	16.6 (0.6)	14.8 (0.6)	P<0.01	17.1 (0.6)	14.3 (0.6)	P<0.01
Insulin, ng/ml	1.39 (0.06)	1.40 (0.06)	P=0.88	1.08 (0.06)	1.72 (0.07)	P<0.01
BST, ng/ml	2.6 (0.1)	15.7 (3.2)	P<0.01	9.1 (2.0)	9.2 (2.5)	P=0.97

[1] Probability of the difference between treatments (A vs B and C vs D) being statistically not different from zero.
[2] Standard errors are shown in parentheses.

Table 7. Least square means for milk yield, milk protein yield, casein yield and plasma IGF-I levels showing the comparisons between treatments where differences were statistically different from zero.

Trait	Treatment[1] (A)	LSM (se)	Treatment (B)	LSM (se)	Pdiff (A-B)[2]
Milk yield, kg/d	S	49.5 (2.0)[3]	I	44.6 (2.0)	P<0.01
	S	49.5 (2.0)	B+I	53.2 (2.0)	P=0.04
	I	44.6 (2.0)	B+I	53.2 (2.0)	P<0.01
Protein yield, kg/d	S	1.38 (0.06)	B+I	1.50 (0.06)	P=0.04
	I	1.30 (0.06)	B+I	1.50 (0.06)	P<0.01
	B	1.42 (0.06)	B+I	1.50 (0.06)	P=0.04
Casein yield, kg/d	S	1.17 (0.05)	B+I	1.28 (0.05)	P<0.01
	I	1.12 (0.05)	B+I	1.28 (0.05)	P<0.01
IGF-I, ng/ml	S	122.5 (43.5)	B	342.3 (43.5)	P<0.01
	S	122.5 (43.5)	B+I	492.2 (28.8)	P<0.01
	I	181.3 (28.8)	B+I	492.2 (28.8)	P<0.01
	B	342.3 (43.5)	B+I	492.2 (28.8)	P<0.01

[1] Treatment code: S = saline infusion, I = insulin and glucose infusion, B = bST administration, B + I = bST administration combined with insulin and glucose infusion.
[2] Probability of the difference between treatments (A vs B) being statistically not different from zero.
[3] Standard errors are shown in parentheses.

CHAPTER 4

Manuscript 3

Effects of Insulin, Recombinant Bovine Somatotropin (rbST) and their Interaction on DMI and Milk Fat Production in Dairy Cows

By

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[5] Dairy and Swine Research and Development Centre contribution number XXX. Please address reprint request to D. Petitclerc.

ABSTRACT

This trial was designed to test the effects of insulin, rbST and their interaction on DMI, milk production and fat yield and composition in dairy cows. Eight Holstein cows (86 ± 10 DIM) were divided in 2 groups and used in 2 replicates of a Latin Square design with 4 animals, 4 periods and 4 treatments: (1) intravenous infusion of saline; (2) infusion of saline and subcutaneous administration of 40 mg of rbST per day; (3) intravenous infusion of 12 mg of insulin per day coupled with glucose infusion; and (4) rbST administration combined with insulin and glucose infusion. The glucose infusion rate was adjusted to maintain normoglycemia. The periods consisted of 14 d; the treatments were administered during the first 6 d and no treatment was administered during the following 8 d (resting phase). Data from the last 3 d of infusion were analysed using the SAS system for mixed models. Fat yield was higher (1.63 vs 1.39 kg/d, $P<0.01$), with a higher content of long chain fatty acids (242.2 vs 176.4 g/milking, $P<0.05$), during rbST administration, regardless of insulin infusion. Insulin infusion decreased fat yield (1.39 vs 1.63 kg/d for the control group, $P<0.01$), mainly through a decrease in long chain fatty acids (179.7 vs 238.9 g/milking for the control group, $P<0.05$), regardless of rbST administration. For DMI, milk yield, fat percent, lactose percent and lactose yield there was a significant interaction ($P<0.01$) between insulin and rbST administration. Results indicate that insulin and rbST do not interact in terms of their effects on milk fat yield and on the production of fatty acids in milk, indicating that these effects are not mediated through the same mechanisms as their effects on milk protein production. The negative effects of insulin on milk fat production are clearly confirmed.

(Key words: insulin, bovine somatotropin, dairy cows)

INTRODUCTION

Humans have used the main components of milk almost since the beginning of recorded

history. Especially in the last decades, milk composition has been increasingly important to milk producers and processors. In addition, the nutritional importance to consumers has been emphasized in a crescent fashion. The introduction of milk pricing based on a component basis and the perception by consumers that animal fats are unhealthy have created new interest in how milk components can be altered to accommodate these trends. Even though milk protein is emerging as the single most important component in milk (Hettinga, 1989), producers receive their income on milk fat yield as well. Besides, changes in milk fat quality relate to rheological properties, which influence numerous aspects of character and quality of manufactured dairy products (Palmquist et al., 1993). As well, the composition of milk fat is important from the point of view of human nutrition. Thus, new technologies devising the maximization of protein yield should be put into perspective regarding milk fat production and composition. This is especially true for studies on the endocrinology of milk synthesis focussing on insulin, since this hormone apparently has the ability to increase milk protein synthesis. Insulin infusion to dairy cows improved the yield of milk protein (Mackle et al., 1999; McGuire et al., 1995; Mackle et al., 2000; Griinari et al., 1997; Léonard and Block, 1997).

However, there is an old and still controversial theory, known as the glucogenic-insulin theory of milk fat depression (McClymont, 1951), stating that there is a decrease in milk fat percentage due to higher levels of insulin. This theory proposes that increased insulin plasma levels channel nutrients to adipose tissue, resulting in a shortage of nutrients at the mammary gland level and, consequently, milk fat depression (Van Soest, 1994). More recently, this theory has been challenged. Lack of an increase in concentrations of glucose and insulin in serum of cows with the greatest decline in percentage of milk fat casts doubt on the ability of the glucogenic theory to explain milk fat depression completely (Gaynor et al., 1995). Bauman and collaborators, during their hyperinsulinemic-euglycemic clamp studies, have not found a significant effect of insulin in reducing milk fat content and yield (McGuire et al., 1995b). From the same group, Griinari et al., 1997a, did not find a significant decrease in milk fat yield, but reported a statistically significant decrease in milk fat content. However, other researchers using the

clamp technique reclaimed that insulin does have a negative effect on milk fat production (Léonard and Block, 1997), although in this case hypoglycemia occurred. Therefore, it is apparent that the milk fat depression theory is still fomenting controversy. This study was designed to further investigate the effects of insulin on milk fat production. The possibility of an interaction between insulin and somatotropin regarding their effects on milk and milk fat production, and on DMI, was tested as well.

MATERIALS AND METHODS

Animals and treatments

Eight Holstein cows averaging 86 ± 10 d in milk were used in 2 replicates of a Latin Square design with 4 cows, 4 periods and 4 treatments. Each period consisted of 14 days; during days 1 to 6 the animals received the treatments (infusion phase) and during days 7 to 14 the animals received no treatment (resting phase). The treatments were (1) infusion of saline (S); (2) infusion of saline and subcutaneous administration of 40 mg of rbST (American Cyanamid Company, Princeton, NJ) per day (B); (3) infusion of 12 mg of insulin (# I 5500, Sigma-Aldrich Canada Ltd, Oakville, Ontario) per day coupled with a variable rate of glucose infusion (I); and (4) subcutaneous administration of 40 mg of rbST per day combined with insulin and glucose infusion (B+I). The schematic representation of the experimental design and the infusion solutions are described in detail elsewhere (Molento *et al.*, accepted). The guidelines of the Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, 1993) were observed for all procedures involving the animals.

Management and measurements

The cows were housed in an artificially lighted and ventilated tie-stall barn with separated mangers that allowed the measurement of individual feed intake. All animals received the same diet in the form of a total mixed ration (TMR) with a 40:60 ratio of

haylage (alfalfa and timothy) + corn silage to grains, formulated according to the requirements (NRC, 1989) and fed at around 110% of their ad libitum intake, in 2 meals per day. During the infusion phase, feed offered and feed refused were weighed daily. Samples of the TMR were collected on d -1 and 0, and 7 and 8 of each period, immediately before and after the infusion; these samples were analysed using traditional wet chemistry procedures for crude protein (method of Kjeldahl, A.O.A.C., 1985), acid detergent fiber (method of Van Soest, 1973), and neutral detergent fiber (method of Goering and Van Soest, 1970). Ether extract, calcium, phosphorus and ash were determined according to A.O.A.C (1985) procedures. Dry matter was determined using a forced draft equipment at 100°C (Koster Crop Tester, Cleveland, Ohio). The average composition of the TMR throughout the experimental periods is presented elsewhere (Molento et al., accepted).

On day 0 an indwelling catheter was installed in the left jugular vein and was maintained for the infusion of treatments throughout the 6 days of the infusion phase. The catheter was connected to an extension that went from the barn to the infusion table, passing through the barn wall. In the case of animals receiving saline, the extension led to a bottle containing saline solution. When animals were receiving insulin and glucose, the extension was connected to 3 shorter extensions; 2 led to bottles containing the glucose solution and 1 led to the insulin solution. The glucose and insulin solutions were kept constantly refrigerated. The pump used for the insulin and saline infusions (Gilson peristaltic pump, miniplus 2, Gilson France SA, Villiers le Bel, France) was kept at a constant flow rate (2 ml/min). There were 2 other pumps (Masterflex peristaltic pump, Cole Palmer Instruments Co., Chicago, IL) which were used for the glucose infusion; each pump served 1 of the 2 cows that were receiving the insulin + glucose infusion within the same period. The flow rate of the glucose infusion was adjusted individually for each cow every two hours, according to blood glucose values read on barn with a glucometer (Accu-Chek Advantage, Boehringer Mannheim, Hoffmann-La Roche Ltd., Quebec, Canada). The objective of the glucose flow rate adjustments was to maintain glycemia within -10% and +20% of baseline values, which were an average of 4 blood

glucose measurements taken on day 0 at 19:00, 21:00, 23:00 and 24:00 h. The infusion started on day 1, at 01:00 h. Blood glucose level of cows receiving insulin and glucose infusion was measured every 20 minutes, until values fell within the expected range, which occurred on average during the first 3 hours. After that, blood glucose was measured every 2 h during the 6 d of infusion, and glucose infusion rate was adjusted accordingly. Details on blood sampling and the laboratory analyses of plasma parameters are presented elsewhere (Molento et al., accepted).

Within each period, milk yield and milk samples for component analyses were taken during days 4, 5 and 6. Days 1, 2 and 3 were considered an adaptation phase (animals received treatments but the production data were not included in the statistical analysis). Double milk samples were taken at each milking. One set of milk samples was sent to the Programme d'Analyse des Troupeaux Laitiers du Québec (PATLQ), where fat and lactose contents were determined with an infrared analyser. The second set was sent to AgriCanada Lennoxville where the determination of fat components was performed.

Milk fatty acids were extracted and methylated according to the method described by Chouinard et al., 1997. Fatty acid methyl ester profiles were measured by gas-liquid chromatography on a Hewlett-Packard 6890 chromatograph (Hewlett-Packard Ltée, Montréal, QC, Canada), according to the method described by Delbecchi et al., 2001. The calculation of fatty acid yield has not taken into account the yield of glycerol.

Statistical analyses

Data were analysed using the proc MIXED of SAS system for mixed models (SAS Institute, 1996). Replicate, period within replicate, rbST, insulin, and day were considered fixed effects. The effects of cow and sample were included in the random statement. The proc MIXED was used because it allows modelling the covariance

structure, which is especially important for the analysis of repeated measures data (Littell et al., 1998). The compound symmetry covariance structure was used for dry matter intake, milk yield, lactose yield and percent, and fat yield and percent, which were analysed by model 1; the variance components covariance structure was used for the statistical analyses of the individual fatty acids, the summation of short chain fatty acids, the summation of long chain fatty acids and their ratio, which were analysed by model 2. Table 1 shows the variance estimates for some of the parameters studied.

$$Y_{ijklmn} = \mu + Rep_i + Per_j(Rep_i) + rbST_k + Ins_l + rbST_k*Ins_l + C_m(Rep_i) + D_n + e_{ijklmno} \quad [1]$$

$$Y_{ijklmn} = \mu + Rep_i + Per_j(Rep_i) + rbST_k + Ins_l + rbST_k*Ins_l + C_m(Rep_i) + D_n + S_o(C_m * Rep_i * Per_j) + e_{ijklmno} \quad [2]$$

In these models Y is the measured parameter, μ is the overall mean, Rep is the replicate effect, Per is the period effect, rbST is the effect of the subcutaneous injection of 40 mg of rbST per day, Ins is the effect of the infusion of 12 mg of insulin per day, C is the cow effect, D is the day effect and S is the sample effect.

RESULTS

All animals completed the study in excellent health and returned to the regular management at Macdonald Campus Dairy Complex of McGill University.

Production traits

Table 2 shows the least square means (LSM) for milk fat yield (kg/d), summation of short chain fatty acids (SCFA) and long chain fatty acids (LCFA) (% and g/milking), and the

ratio SCFA:LCFA. For these traits the interaction between insulin and rbST was not statistically significant (P>0.05), therefore the main effects of these hormones are shown. During rbST administration, fat yield was higher (P<0.01), percentage of total milk fat content represented by SCFA was lower (P=0.01), summation of SCFA on a yield basis was not different (P>0.05), percentage of total milk fat content represented by LCFA was higher (P<0.01), summation of LCFA on a yield basis was also higher (P=0.01), and the ratio SCFA:LCFA, on a percentage basis as well as on a yield basis, was significantly decreased by rbST (P<0.01). Insulin infusion decreased fat yield (P<0.01), did not alter percentage of total milk fat content represented by SCFA (P>0.05), nor the summation of SCFA on a yield basis (P>0.05), decreased the percentage of total milk fat content represented by LCFA (P<0.01), and the summation of LCFA on a yield basis (P<0.05), and increased the ratio SCFA:LCFA, which again was virtually the same on a percentage basis as well as on a yield basis (P<0.01).

For DMI, milk yield, fat content, lactose content and lactose yield, there was a significant interaction between insulin and rbST administration (P<0.01). Figures 1, 2 and 3 show the effects of insulin infusion within the two levels of rbST on DMI, milk yield, and fat content, respectively. The administration of 40 mg of rbST per day decreased the impact of insulin infusion on DMI (Figure 1), although significant differences were only found for the comparison between saline and all other treatments (Table 3). The infusion of 12 mg of insulin per day in the absence of rbST administration caused a decrease in yield of milk and lactose, but the same infusion of insulin in the presence of 40 mg of rbST per day leads to an increase; the LSM for the comparisons which were statistically significant are shown in table 3. The graphical representation of the interaction between treatments for lactose yield presents the same pattern as that for milk yield (Figure 2). The ability of rbST to increase milk fat content observed during this trial was lost when there was simultaneous infusion of insulin (Table 3, Figure 3). Even though the interaction between rbST and insulin was significant in terms of lactose content, none of the comparisons were of statistical significance, being the LSM 4.80 ± 0.03, 4.75 ± 0.03, 4.76 ± 0.03 and 4.79 ± 0.03 % for saline, insulin, rbST and simultaneous insulin and rbST treatments,

respectively.

For most milk fatty acids analysed, there was no interaction between the effects of exogenous insulin and rbST. The LSM for these fatty acids, both as a percentage of total fat content and on a yield basis (g/milking), showing the overall effects of rbST or insulin administration, are presented (Table 4). Effects on fatty acids that were statistically significant on a yield basis are highlighted in Figure 4. In summary, insulin infusion had significant effects on the yields of only three fatty acids, increasing the production of $C_{14:1}$, and decreasing $C_{18:0}$ by + 30.2, - 41.4 %, respectively; rbST increased the secretion of C20:1 by +24.8 %.

The only fatty acids where there was a significant interaction between insulin and rbST treatments were $C_{18:2}$ (%) and $C_{18:3}$ (%). The only comparison within the interaction which showed statistical significance was for $C_{18:3}$ (%), between saline and insulin infusion, which had a LSM of 0.37 ± 0.01 and 0.30 ± 0.01, respectively. Although the interaction between insulin and rbST had a significant effect ($P<0.05$) on the percentage of $C_{18:2}$ in milk, none of the comparisons was significant; being the LSM 1.91 ± 0.08, 1.63 ± 0.08, 1.81 ± 0.08 and 1.88 ± 0.08 % for saline, insulin, rbST, and simultaneous insulin and rbST treatments, respectively.

DISCUSSION

Recombinant bST changed the response in DMI when animals received insulin infusion, the decline in DMI caused by insulin being more severe in the absence of rbST. The only statistically significant differences were for comparisons of the effect of saline versus the other treatments; when cows received saline, they ate significantly more than when they received rbST, insulin, or both combined. Recombinant bST usually leads to an increase in DMI (Peel and Bauman, 1987), being this increase in DMI related to rbST effects on milk yield. Thus, an increase in DMI was probably limited by the timeframe of the present experiment. Experiments with either short-term (Peel and Bauman, 1987) or

medium-term (de Boer, 1991) rbST treatment show no effect on DMI, and the negative effect of rbST on DMI observed here was unexpected. Hereford cows showed a reduction in DMI during bST treatment; the authors cited changes in other hormones as a possible cause, although they did not specify which hormones (Bines et al, 1980). The effect of rbST on DMI might be related to the extent of fat mobilization, as seen by the magnitude of the increase in fat yield caused by rbST (240 extra g of milk fat per day). The composition of milk fat also points to an extensive mobilization, since the overall increase in milk fat seems to be due mainly to the increase in LCFA. Body weight and body condition score were monitored, but experimental periods were too short to use these parameters to estimate fat mobilization. The decrease in DMI caused by insulin and glucose infusion has been reported before (Griinari et al., 1997a); this effect was expected since the glucose infusion supplies part of the energy requirements of the animals. With an overall average of 2.5 kg of glucose per day during the clamp period, animals on this treatment were receiving extra 9.3 Mcal of energy from glucose intravenous infusion, considering 673 kcal/mol glucose (Milligan, 1971).

Milk and lactose yield responses to the interaction between insulin and rbST showed the same trend as that observed by the interaction on circulating IGF-I levels (Molento et al., accepted). Lactose yield and consequently milk yield are not directly related to insulin in the mammary gland, since lactose synthesis by the mammocytes is not regulated by insulin (Hove, 1978). However, our results show that insulin is able to modify the response of these variables to rbST administration. Insulin infusion led to a greater response in the yield of milk and lactose when rbST was administered. In other words, this illustrates the decreased ability of bST to increase milk yield when insulin levels are lower, which in turn might be related to the decreased secretion of IGF-I, referred to as the uncoupling of the bST-IGF-I axis (Léonard and Block, 1997; Burton et al., 1994).

The increase in milk fat yield observed with rbST treatment, regardless of insulin effects, has been reported previously. For example, Bitman et al., 1984, observed a 41 % increase in milk fat yield when animals received rbST. In their work, as in the results presented

here, the overall changes of milk lipids were consistent with the concept that adipose tissue reserves were mobilized in response to rbST treatment and that these mobilized lipids were the major carbon source for the increase of milk fat secretion. As reported here, administration of rbST increased production of total milk fat (Lynch et al., 1992), these changes in composition or physical properties of milk fat being within the range of normal variation found due to stage of lactation.

The unquestionable decrease in milk fat yield caused by insulin infusion, regardless of rbST treatment, supports the glucogenic-insulin theory of milk fat depression (McClymont, 1951). A decrease in fat yield has been reported before during the insulin clamp (Léonard and Block, 1997), although the hypoglycemia due to the insulin infusion prevented a clear conclusion. The effect of insulin in reducing milk fat yield is confirmed in this study, without the confounding effects of hypoglycemia. This confirmation is in contradiction with recent literature. Six factors are of special relevance when comparing the results reported here with those of Griinari et al, 1997a, and McGuire et al., 1995: (1) the experimental design of the present work, which was a Latin Square, represents the first time that the effects of the insulin clamp are studied without the confounding effects of DIM; (2) although there were three different trials, the number of animals used by those researchers in each experiment was smaller; (3) the statistical analysis has never been as sensitive as the one employed in the present work, involving the use of mixed models and the testing of different covariance structures; (4) the changes in levels of blood insulin induced here represent a more physiological variation, where insulin infusion led to an increase of 62.8 % in blood insulin, as opposed to previous studies (e.g. the clamp performed by Griinari et al., 1997a, attained an increase of blood insulin in the order of 425 % the baseline level); (5) experimental animals were earlier in lactation than most clamp studies (e.g. Griinari et al., 1997a, used cows 184 DIM); and (6) most of the studies with the clamp which did not find a significant decrease in milk fat yield present results after four days of insulin infusion, while the results here are from samples collected up to d 6 of the clamp. These factors combined, giving emphasis to the power of the experimental design and statistical analysis, might explain why a significant effect

of insulin on milk fat yield was not found in previous studies, especially considering that a numerical decrease was consistently observed.

The response in terms of fat yield showed a completely different reaction to the interaction between the hormones tested if compared to the response found for protein and casein yield (Molento et al., accepted). There was no effect of the interaction on fat yield. These findings suggest that the effects of treatments on fat yield are not mediated through plasma IGF-I. In vitro results support this theory, since IGF-I did not increase the rate of lipolysis in ruminant adipose tissue (Houseknecht, 1996).

A decrease in fatty acids up to C_{14} is generally responsible for the milk fat depression reported when animals are on a high concentrate diet (Palmquist et al., 1993). However, the milk fat depression observed here is due to a decrease in LCFA, instead of a decrease in SCFA. Thus, the mechanism for the fat yield reduction observed with the insulin clamp is not the same as with high concentrate diets. An important difference between diet changes and insulin infusion is that in the first case, as insulin increases in response to a diet richer in concentrate, bST decreases (Gaynor et al., 1995). It is not known what happens to bST levels when higher insulin blood levels are attained through the hyperinsulinemic-euglycemic clamp, but looking at blood profile for the group of animals receiving only insulin, blood bST did not seem to be significantly altered for the duration of the clamp (Molento et al, accepted). Therefore, an interaction between insulin and bST might be central to the explanation for the responses in terms of fat composition response to insulin. These results confirm previously observed changes during the clamp: the balance between de novo synthesis and preformed fatty acids in milk fat shifted toward those synthesized de novo (Griinari et al., 1997a).

The overall decrease in milk LCFA due to insulin infusion has been reported before (Griinari et al., 1997a); nevertheless, it is the first time that the interaction between insulin and rbST is tested regarding the effects on milk fat composition. For all individual fatty acids tested this interaction was not statistically significant, thus the discussion is

centred on overall effects of either hormone treatment. Although the percentages of $C_{4:0}$ and $C_{6:0}$ were significantly reduced by insulin infusion, the statistical analyses on a yield basis did not show any significant effect of the insulin clamp. Thus, the decreased percentage of these fatty acids is probably an artefact of changes on the amount of other individual fatty acids, and this example illustrates the importance of comparing milk fatty acids on a yield basis. For this reason, discussion is focussed on significant changes observed on a yield basis. The lack of an effect of insulin infusion on the production of $C_{4:0}$ and $C_{6:0}$ is in contradiction with results reported by Griinari et al., 1997a. Yields of $C_{10:0}$ and $C_{12:0}$, reported previously to increase with the insulin clamp (Griinari et al., 1997a), were not statistically influenced by insulin in this study. These differences might be due to the fact that individual SCFA are present in low concentrations in milk, being the interpretation of their summation more meaningful. Griinari et al., 1997a, report an increase in $C_{14:1}$ and a decrease in $C_{18:0}$ due to insulin infusion which were confirmed in this study (Figure 4). The bulk of rbST research indicates that it does not alter the proportions of fatty acids in milk (Burton et al., 1994). The increase in $C_{20:1}$ observed here (Figure 4, Table 4) is probably correlated to a greater fat mobilization in the rbST treated animals, which in turn might be correlated to the decreased DMI.

CONCLUSION

The study of the interaction between insulin and rbST in dairy cows has provided original information, which corroborates to the understanding of the mechanisms through which insulin controls dairy production traits. A significant interaction between insulin and rbST was observed for DMI, milk yield, fat content and lactose yield, elucidating that the changes induced in these variables by rbST depend on the levels of insulin, and vice-versa. Furthermore, the yields of milk and lactose responded to the interaction between insulin and rbST with the same pattern as circulating IGF-I levels, indicating IGF-I as a possible mediator of this interaction.

Adding another piece of information on the current controversy regarding insulin effects on milk fat production, our results clearly show a significant reduction in milk fat yield due to insulin infusion. It is also observed that there is no interaction between insulin and rbST on milk fat yield and on the production of all individual fatty acids in milk. Therefore, it is concluded that the insulin control of the yields of total fat as well as individual fatty acids by the mammary gland is independent of ST levels. In addition, these variables are not mediated by the same mechanisms as milk lactose and protein, since the latters responded significantly to the interaction between insulin and rbST. These results suggest that the involvement of IGF-I as a regulatory factor is not operating in terms of milk fat.

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Figure 1. The effects of insulin + glucose infusion on DMI within two different levels of recombinant bovine somatotropin (rbST). The interaction between treatments was significant; refer to Table 3 for details on the significance of comparisons.

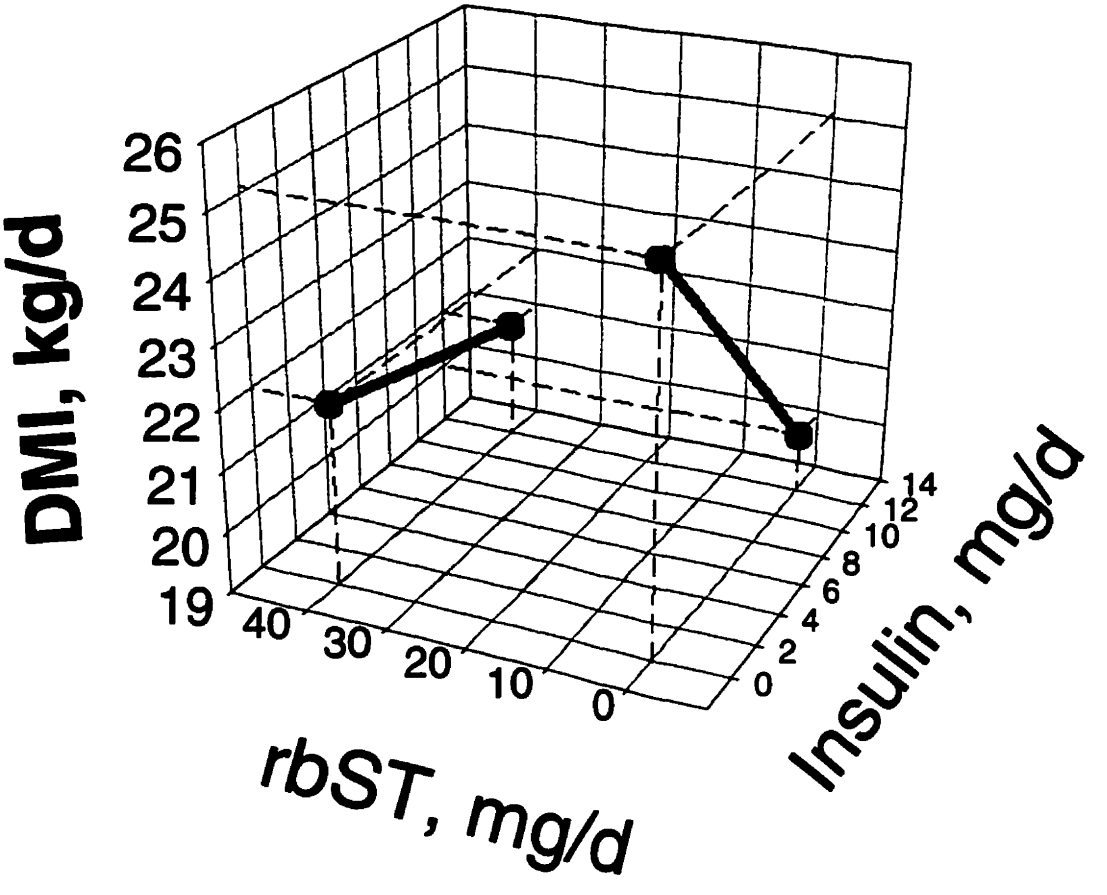

Figure 2. The effects of insulin + glucose infusion on milk yield within two different levels of recombinant bovine somatotropin (rbST). The interaction between treatments was significant; refer to Table 3 for details on the significance of comparisons.

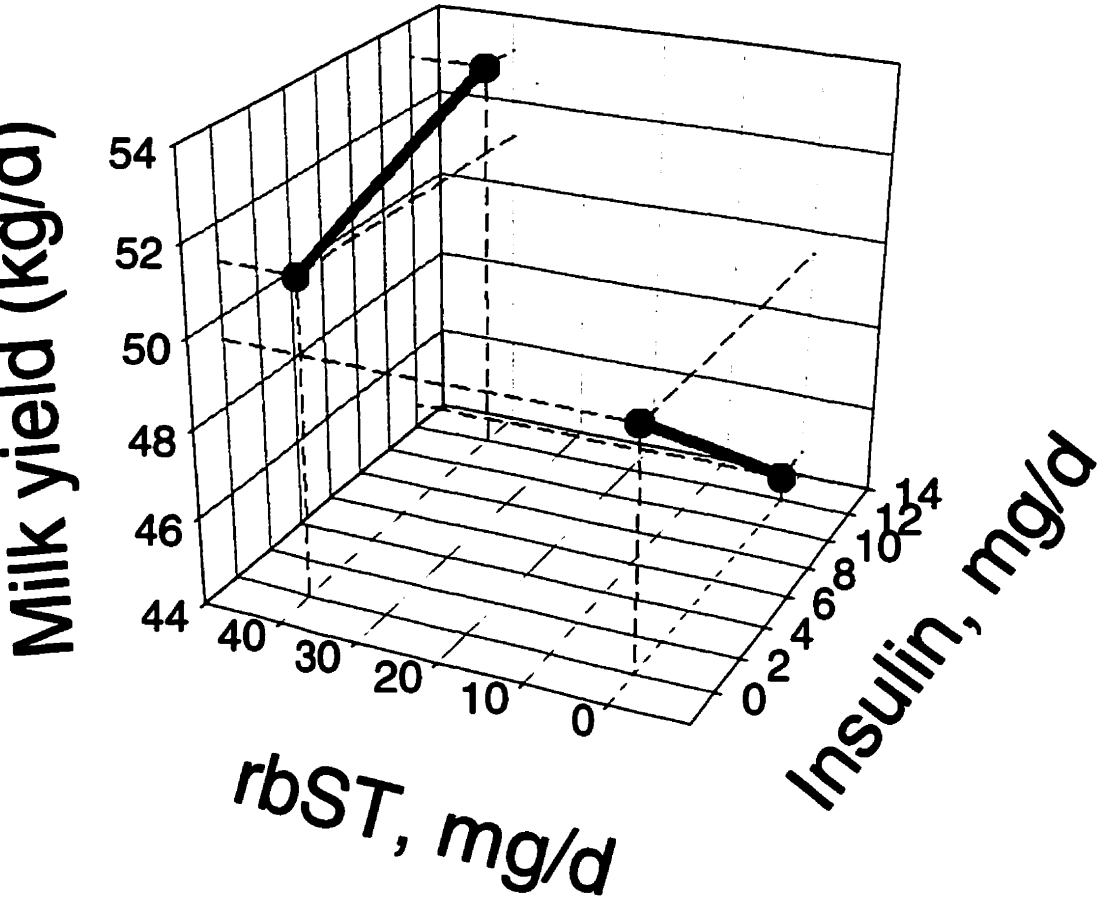

Figure 3. The effects of insulin + glucose infusion on milk fat content within two different levels of recombinant bovine somatotropin (rbST). The interaction between treatments was significant; refer to Table 3 for details on the significance of comparisons.

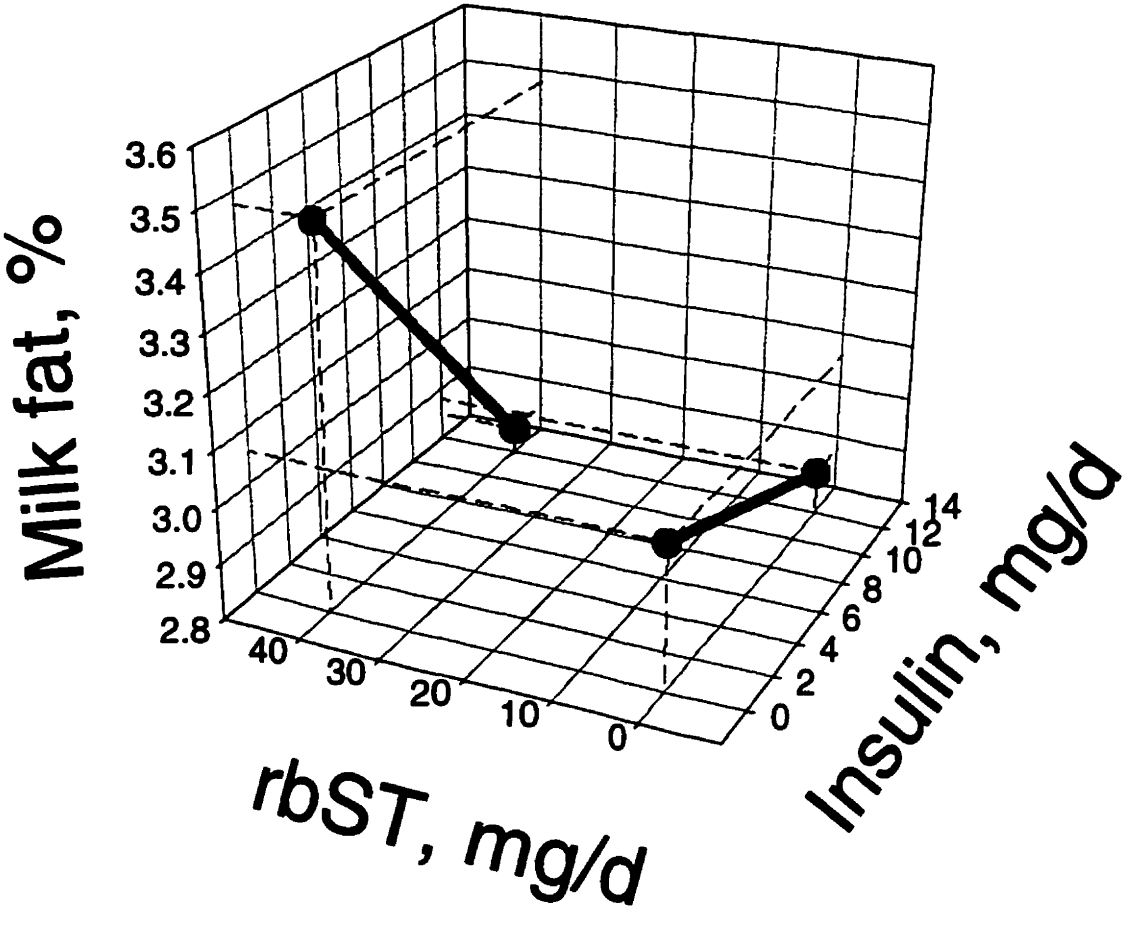

Figure 4. Percentage change for fatty acid secretion (g/milking) in milk, showing the fatty acids where there was a significant overall effect of either insulin or rbST.

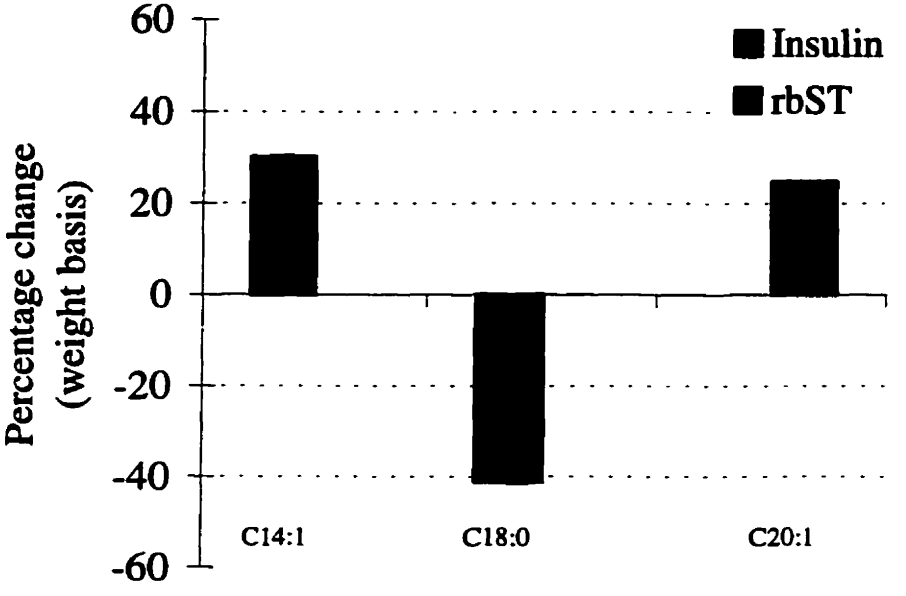

Table 1. Variance estimates for dry matter intake, milk yield, lactose yield, lactose percent, fat yield, and fat percent.

Parameter	Cow variance	Sample variance	CS Covariance[1]	Residual variance
Dry matter intake, kg/d	8.44	-	0.07	12.97
Milk yield, kg/d	24.98	-	3.28	16.12
Lactose yield, kg/d	0.067	-	-0.003	0.038
Lactose, %	0.0076	-	0.0002	0.0040
Fat yield, kg/d	0.066	-	0.003	0.045
Fat, %	0.164	-	0.002	0.010
SCFA[2], g/milking	803.3	2184.1	-	0.9
SCFA, %	2.54	17.39	-	0.02
LCFA[3], g/milking	2060.7	2920.7	-	0.6
LCFA, %	0.97	27.67	-	0.02

[1]The compound symmetry covariance structure CS was used for dry matter intake, milk yield, lactose yield and percent, and fat yield and percent; the variance components VC covariance structure was used for the short and long chain fatty acids analysis.
[2] SCFA = Summation of Short Chain Fatty Acids, from 4 to 15 carbons.
[3] LCFA = Summation of Long Chain Fatty Acids, from 17 to 20 carbons.

Table 2. Least square means for milk fat yield (kg/d), SCFA[1], LCFA[2], and their ratio, both as a percentage of total fat content (%) and on a yield basis (g/milking), showing overall effects of rbST or insulin administration.

Trait	bST			Insulin		
	0 mg/d[3]	40 mg/d	Pdiff[4]	0 mg/d	12 mg/d	Pdiff
Fat yield, kg/d	1.39 (0.10)	1.63 (0.10)	P<0.01	1.63 (0.10)	1.39 (0.10)	P<0.01
SCFA content, %	27.3 (1.4)	22.6 (1.4)	P=0.01	23.3 (1.4)	26.5 (1.4)	NS[5]
SCFA, g/milking[6]	128.7 (17.8)	134.4 (17.8)	NS	127.1 (17.3)	135.9 (18.5)	NS
LCFA content, %	36.4 (1.6)	43.9 (1.6)	P<0.01	43.8 (1.6)	36.6 (1.6)	P<0.01
LCFA, g/milking	176.4 (23.5)	242.2 (23.5)	P=0.01	238.9 (23.1)	179.7 (24.3)	P=0.03
Ratio SCFA:LCFA, Based on %	0.78 (0.06)	0.54 (0.06)	P<0.01	0.54 (0.06)	0.78 (0.06)	P<0.01
Ratio SCFA:LCFA, based on yield	0.79 (0.06)	0.54 (0.06)	P<0.01	0.54 (0.06)	0.80 (0.07)	P<0.01

[1] SCFA = Summation of Short Chain Fatty Acids, from 4 to 15 carbons.
[2] LCFA = Summation of Long Chain Fatty Acids, from 17 to 20 carbons.
[3] Standard errors are shown in parentheses.
[4] Probability of the difference being statistically not different from zero.
[5] NS = not statistically significant (P>0.05).
[6] Cows were milked 3 times per day.

Table 3. Least square means for dry matter intake (kg/d), milk yield (kg/d), fat content (%), lactose content (%) and lactose yield (kg/d), where the interaction between insulin and rbST was significant, showing the comparisons where differences were statistically different from zero.

Trait	Treatment[1] (A)	LSM (se)	Treatment (B)	LSM (se)	Pdiff (A-B)[2]
DMI, kg/d	S	25.1 (1.28)[3]	I	20.0 (1.28)	P<0.01
	S	25.1 (1.28)	B	22.0 (1.28)	P=0.04
	S	25.1 (1.28)	B+I	21.0 (1.28)	P<0.01
Milk yield, kg/d	S	49.5 (2.0)	I	44.6 (2.0)	P<0.01
	S	49.5 (2.0)	B+I	53.2 (2.0)	P=0.04
	I	44.6 (2.0)	B+I	53.2 (2.0)	P<0.01
Fat content, %	S	3.06 (0.16)	B	3.48 (0.16)	P<0.01
	B	3.48 (0.16)	B+I	2.84 (0.16)	P<0.01
Lactose yield, kg/d	S	2.37 (0.10)	I	2.14 (0.10)	P<0.01
	I	2.14 (0.10)	B+I	2.54 (0.10)	P<0.01

[1] Treatment code: S = saline infusion, I = insulin and glucose infusion, B = bST administration, B + I = bST administration combined with insulin and glucose infusion.
[2] Probability of the difference between treatments (A vs B) being statistically not different from zero.
[3] Standard errors are shown in parentheses.

Table 4. Least square means for fatty acids both as a percentage of total fat content (%) and on a yield basis (g/milking), showing overall effects of rbST or insulin administration.

Trait	BST			Insulin		
	0 mg/d[1]	40 mg/d	Pdiff[2]	0 mg/d	12 mg/d	Pdiff
C4:0, %	4.16 (0.25)	3.95 (0.25)	NS[3]	4.37 (0.25)	3.74 (0.25)	P<0.01
C4:0, g/milking[4]	19.7 (2.9)	22.9 (2.9)	NS	24.0 (2.9)	18.6 (3.0)	NS
C6:0, %	1.96 (0.14)	1.71 (0.14)	NS	1.81 (0.14)	1.86 (0.14)	NS
C6:0, g/milking	9.2 (1.5)	10.3 (1.5)	NS	10.1 (1.5)	9.3 (1.6)	NS
C8:0, %	1.12 (0.09)	0.93 (0.09)	NS	0.94 (0.09)	1.11 (0.09)	NS
C8:0, g/milking	5.21 (0.96)	5.68 (0.96)	NS	5.26 (0.93)	5.63 (0.99)	NS
C10:0, %	2.65 (0.27)	2.11 (0.27)	NS	2.02 (0.28)	2.74 (0.28)	P=0.04
C10:0, g/milking	12.4 (2.4)	13.0 (2.4)	NS	11.2 (2.4)	14.2 (2.5)	NS
C12:0, %	3.29 (0.32)	2.51 (0.32)	P=0.04	2.36 (0.33)	3.44 (0.33)	P<0.01
C12:0, g/milking	15.5 (2.8)	15.4 (2.8)	NS	13.1 (2.8)	17.8 (3.0)	NS
C14:0, %	11.36 (0.59)	9.24 (0.59)	P<0.01	9.72 (0.60)	10.88 (0.60)	NS
C14:0, g/milking	53.9 (7.2)	55.0 (7.2)	NS	52.6 (7.0)	56.4 (7.5)	NS

C14:1, %	1.55 (0.12)	1.31 (0.12)	P=0.05	1.19 (0.13)	1.67 (0.13)	P<0.01
C14:1, g/milking	7.3 (0.8)	7.2 (0.8)	NS	6.3 (0.7)	8.2 (0.8)	P=0.04
C15:0, %	1.17 (0.11)	0.87 (0.11)	P<0.01	0.91 (0.11)	1.13 (0.11)	P=0.03
C15:0, g/milking	5.7 (0.6)	4.7 (0.6)	NS	4.9 (0.6)	5.5 (0.6)	NS
C16:0, %	34.3 (0.84)	30.9 (0.84)	P<0.01	30.7 (0.85)	34.5 (0.85)	P<0.01
C16:0, g/milking	159.4 (19.6)	179.9 (19.6)	NS	168.0 (19.2)	171.3 (20.3)	NS
C16:1, %	2.03 (0.25)	2.43 (0.25)	NS	1.96 (0.26)	2.51 (0.26)	NS
C16:1, g/milking	9.5 (1.3)	12.4 (1.3)	NS	10.2 (1.3)	11.7 (1.4)	NS
C17:0, %	0.55 (0.02)	0.59 (0.02)	NS	0.59 (0.02)	0.55 (0.02)	NS
C17:0, g/milking	2.64 (0.30)	3.17 (0.30)	NS	3.17 (0.29)	2.65 (0.31)	NS
C18:0, %	9.2 (0.78)	9.6 (0.78)	NS	11.5 (0.78)	7.3 (0.78)	P<0.01
C18:0, g/milking	45.5 (8.0)	54.7 (8.0)	NS	63.1 (7.9)	37.0 (8.2)	P<0.01
C18:2, g/milking	8.6 (1.2)	10.5 (1.2)	NS	10.4 (1.1)	8.7 (1.2)	NS
C18:3, g/milking	1.63 (0.20)	1.96 (0.20)	NS	2.00 (0.20)	1.59 (0.21)	NS
C20:1, %	0.70 (0.04)	0.71 (0.04)	NS	0.63 (0.04)	0.77 (0.04)	P=0.01

C20:1, g/milking	3.23 (0.38)	4.03 (0.38)	P=0.05	3.51 (0.37)	3.75 (0.39)	NS

[1] Standard errors are shown in parentheses.
[2] Probability of the difference being statistically not different from zero.
[3] NS = not statistically significant (P>0.05).
[4] Cows were milked 3 times per day.

GENERAL CONCLUSIONS

Studies were conducted to probe the effect of insulin on milk production and milk components secretion by using two different approaches: one based on the potential induction of endogenous insulin secretion through dietary modifications, and the other by direct intravenous infusion of insulin while maintaining euglycemia.

The putative insulin secretagogue fed in the first trial was Calcium propionate (Ca prop). Unfortunately, in order to avoid the risk of feeding insufficient amounts of Ca prop, the maximization of the daily dose led to a decrease in DMI, which prevented successful augmentation of insulin secretion in the Ca prop group. This decrease in DMI could have been caused by the combination of the low Ca prop palatability and the negative feedback this VFA exerts on the control of DMI. The fact that none of the production traits were affected points to an increase in feed efficiency by the Ca prop-fed animals, considering that DMI was lower than in the control. Moreover, regardless of the decrease in DMI, Ca prop was capable of increasing milk protein percentage. The mechanism through which the increase in milk protein percentage was elicited is not clear, since the hypothesized increase in plasma insulin was not demonstrated. Either there is another mechanism involved, or the experimental design, especially regarding number of animals used and quantity of Ca prop fed, was not sensitive enough to detect an effect on insulin as being the mediator of the observed changes. Regardless of the increase in protein percentage in milk, protein yield (kg/d) did not change, following the same pattern as milk yield. Results show that the level of Ca prop fed increased the concentration of rumen propionate relative to acetate and butyrate concentrations at 2 h after feeding, but this effect was not sustained until 4 h after feeding. The question remains as to whether there is a Ca prop inclusion rate in the diet of dairy cows that will not decrease DMI while still significantly altering the molar ratio of VFA in the rumen. Although insulin responsiveness to insulin secretagogues is lower during lactation, there are two reasons for searching for feed ingredients that lead to higher plasma insulin during lactation: (1) there is a certain degree of responsiveness to insulin, which tends to increase as lactation progresses and (2) the ever increasing number of studies showing insulin's ability to improve milk protein production. This work shows that Ca prop is a potential feed

ingredient to increase protein percentage in milk, although the underlying mechanism is not clear. Further research is needed to determine the effects of Ca prop on milk, fat and protein yields without the confounding effects of a decreased DMI. The value of Ca prop as a feed ingredient to increase both protein percentage and yield will depend on the possibility of feeding an amount of Ca prop that is low enough not to affect negatively DMI and yet high enough to significantly stimulate the synthesis of milk protein by the mammary gland.

The next approach made in this PhD research was to infuse insulin intravenously in lactating dairy cows. It employed the hyperinsulinemic-euglycemic clamp technique, which has been successfully utilised to study the effects of insulin administration while maintaining normoglycemia. Our results confirm that, in the establishment of a hyperinsulinemic-euglycemic clamp in dairy cows, it is essential to continuously monitor blood glucose levels and adjust the rate of glucose infusion accordingly. Furthermore, the results provide direct evidence that rbST injection induces a resistance to insulin effects on glucose utilisation by peripheral tissues.

All effects of insulin reported from studies with the clamp in the literature have been consistently confounded with DIM; all previous studies with the hyperinsulinemic-euglycemic clamp in cows that succeeded in maintaining normoglycemia were designed so that the control period was a baseline period before the clamp started. Hence, when cows received insulin infusion, they were also later in their lactation with reference to the baseline period. It is known that, as lactation progresses, the percentages of protein and fat in milk tend to increase and the yields of milk and components tend to decrease. The same is true for plasma levels of insulin, bST and IGF-I, which tend to increase, decrease and increase, respectively, as lactation progresses. An important feature of the results presented in this study is that they were obtained using a Latin Square design, so they are adjusted for the effect of time. Some effects of insulin on production traits were confirmed, like insulin's ability to increase milk protein content; others are in conflict with previous reports, as for instance the effects of the insulin clamp on milk fat yield.

Insulin is able to increase milk protein content, and this effect is even more pronounced when we consider casein content; insulin significantly reduced NPN in the form of urea as well. We also observed that rbST treatment significantly reduced casein and urea contents in milk, and caused a marginal decrease in total protein content. This might be related to the short timeframe of rbST administration used in this study and to the effects observed on DMI.

The interaction between insulin and rbST on production traits were fully characterized in this study. The increase in plasma insulin concentration attained with the clamp was able to significantly alter the responses to rbST in terms of milk, lactose, protein and casein yield, and lactose content. The milk, lactose and protein yield, as well as casein yield responses showed great similarity to the interaction between insulin and rbST on circulating IGF-I levels. Insulin infusion led to a greater response in the yield of milk, lactose, protein and casein when rbST was administered. In other words, this illustrates the decreased ability of bST to increase milk, lactose, protein and casein yield when insulin levels are lower, which in turn might be related to the decreased secretion of IGF-I, referred to as the uncoupling of the bST-IGF-I axis. It is thought that IGF-I is related to protein synthesis in the mammary gland, and the similarity between IGF-I levels and milk/protein yield responses to treatments is another indication of a strong role of IGF-I on milk protein synthesis.

The mechanism of action of the insulin clamp on milk protein synthesis is not fully understood. Although a direct effect of insulin on milk yield has not been reported, it is likely that insulin participates in bST stimulation of milk production and is probably mediated indirectly by IGF-I. The bST to insulin ratio might be a better indicator to predict IGF-I synthesis by the liver than bST levels alone. This ratio could explain variations in the ability of bST to stimulate liver IGF-I production under different nutritional and physiological conditions. Therefore, increases in milk protein due to insulin might be (a) direct, via the effects of insulin either on the stimulation of mammary

epithelial cell proliferation or on AA transport systems on mammary cells; (b) indirect, via increases in IGF-I combined with changes in insulin-like growth factor binding protein levels or also via changes in bST receptors in the liver affecting ST stimulation of IGF-I release or (c) a combination of both.

Subsequently, data from this trial was evaluated regarding the effects of the hormone treatments on DMI and milk fat yield. Recombinant bST changed the response in DMI when animals received insulin infusion, the decline in DMI caused by insulin being more severe in the absence of rbST. The only statistical differences were for comparisons of the effect of saline versus the other treatments; when cows received saline they ate significantly more than when they received rbST, insulin, or both combined. The effect of rbST on DMI might be related to the extent of fat mobilization, as seen by the magnitude of the increase in fat yield caused by rbST. The composition of milk fat also points to an extensive mobilization, since the overall increase in milk fat seems to be due mainly to the increase in LCFA. The decrease in DMI caused by insulin and glucose infusion has been reported before and it is expected since the glucose infusion supplies part of the energy requirements of the animals.

The increase in milk fat yield observed with rbST treatment, regardless of insulin effects, has been reported previously. Again, the overall changes of milk lipids were consistent with the concept that adipose tissue reserves were mobilized in response to rbST treatment and that these mobilized lipids were the major carbon source for the increase of milk fat secretion. The unquestionable decrease in milk fat yield caused by insulin infusion, regardless of rbST treatment, supports the controversial glucogenic-insulin theory of milk fat depression. This confirmation is in contradiction with recent literature. Six factors are of special relevance when considering the results reported here: (1) the experimental design of the present work, which was a Latin Square, represents the first time that the effects of the insulin clamp are studied without the confounding effects of DIM; (2) the number of animals used was greater than in previous work with the clamp; (3) the statistical analysis has never been as sensitive as the one employed in the present

work, involving the use of mixed models and the testing of different covariance structures; (4) the changes in levels of blood insulin induced here represent a more physiological variation, where insulin infusion led to an increase of 62.8 % in blood insulin, as opposed to previous studies reaching up to 500 % increases; (5) experimental animals were earlier in lactation than in most clamp studies; and (6) most of the studies with the clamp which did not find a significant decrease in milk fat yield presented results after four days of insulin infusion, while the results here are from blood samples collected on d 6 of the clamp. The cumulative effects of these factors, given the power of the experimental design and statistical analysis, might explain why previous studies failed to find a significant effect of insulin on milk fat yield, especially considering that a numerical decrease was consistently observed.

The response in terms of fat yield showed a completely different reaction to the interaction between the hormones tested if compared to the response found for protein and casein yield. There was no effect of the interaction on fat yield. These findings suggest that the effects of treatments on fat yield are not mediated through plasma IGF-I. In vitro results support this theory, since IGF-I did not increase the rate of lipolysis in ruminant adipose tissue.

It is the first time that the interaction between insulin and rbST is tested regarding the effects on milk fat composition. For all individual fatty acids tested, this interaction was not statistically significant. These results suggest that apparently the involvement of IGF-I as a regulatory factor is not operating in terms of milk fat yield and composition. Insulin caused an increase in $C_{14:1}$ and a decrease in $C_{18:0}$, which have been reported previously. The bulk of rbST research indicates that it does not alter the proportions of fatty acids in milk. The increase in $C_{20:1}$ observed here is probably correlated to a greater fat mobilization in the rbST treated animals, which in turn might be correlated to the decreased DMI.

In summary, the present research program was successful in further expanding knowledge of the physiology of lactation in dairy cows. This thesis elucidates the effects of the interaction between insulin and rbST on DMI, milk and milk component yield and plasma IGF-I. Although some of these effects were already suspected through comparison of different reports, it is the first time an experiment is designed to test this interaction. Some effects of insulin on milk component synthesis were confirmed, like its ability to influence positively the synthesis and secretion of milk protein. The issue of insulin acting as a negative regulator of milk fat synthesis, which has been controversial in the literature, was clearly demonstrated here. Results provide evidence on the role of insulin in milk fat depression.

Further studies in this field should be directed towards the search of feed ingredients capable of stimulating endogenous insulin release in lactating dairy cows. Research for appropriate levels of Ca prop are warranted. Employing these ingredients in farms that are routinely on an rbST program would be especially beneficial.

STATEMENT OF ORIGINALITY

To the best of the author's knowledge, the following information contained in this thesis constitute an original contribution to the scientific literature:

- This thesis contains two manuscripts that report for the first time the effects of the hyperinsulinemic-euglycemic clamp on dry matter intake, Insulin-like Growth Factor I and production traits tested with a Latin Square design.

- Direct in vivo proof of the decrease in peripheral glucose utilization caused by recombinant bovine somatotropin (rbST).

- There is a statistically significant interaction between rbST and concentration of insulin in blood on dry matter intake.

- Combination of exogenous rbST and insulin leads to higher circulating levels of IGF-I than any of the two hormones given alone.

- There is a statistically significant interaction between rbST and insulin on the following production traits: milk yield, milk fat percentage, milk protein yield, milk casein yield, milk lactose percentage and yield.

- Insulin significantly increases milk protein and casein percentage and decreases urea in milk when the treatment effect is not confounded with days in lactation.

- Insulin significantly decreases fat yield when the treatment effect is not confounded with days in lactation, confirming the controversial glucogenic-insulin theory of milk fat depression.

- Milk fat depression caused by insulin produces a decrease in the content of long chain fatty acids in milk, as opposed to milk fat depression induced by diet, which causes a decrease in the content of short chain fatty acids in milk.

- The feeding of 300 g of calcium propionate per day to lactating dairy cows leads to a decrease in DMI and to an increase in milk protein percentage.

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