Bovine Growth Hormone and Milk Fat Synthesis: from the Body to the Molecule

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ABSTRACT: Injection of bovine growth hormone (bGH) to lactating dairy cows increases milk yield and yields of milk components including fat. It is generally believed that most of the anabolic effects derived from bGH in animal tissues are primarily mediated by IGF-1. IGF-1 is a strong anabolic peptide in the plasma of animals and exerts mitogenic and metabolic effects on target cells. Contrary to most protein hormones, the majority of IGF-1 in circulation is bound to the binding proteins (IGFBPs) which are known to be responsible for modifying the biological actions of IGF-1, thus making determinations of IGF-1 actions more difficult. On the other hand, fat is a major milk component and the greatest energy source in milk. Currently, the fat content of milk is one of the major criteria used in determining milk prices. It has been known that flavor and texture of dairy products are mainly affected by milk fat and its composition. Acetyl-CoA carboxylase (ACC) is the rate limiting enzyme which catalyzes the conversion of acetyl-CoA to malonyl-CoA for fatty acid synthesis in lipogenic tissues of animals including bovine lactating mammary glands. In addition to the short-term hormonal regulation of ACC by changes in the catalytic efficiency per enzyme molecule brought about by phosphorylation and dephosphorylation of the enzyme, the long-term hormonal regulation of ACC by changes in the number of enzyme molecules plays an essential role in control of ACC and lipogenesis. Insulin, at supraphysiological concentrations, binds to IGF-1 receptors, thereby mimicking the biological effects of IGF-1. The receptors for insulin and IGF-1 share structural and functional homology. Furthermore, epidermal growth factor increased ACC activity in rat hepatocytes and adipocytes. Therefore, it can be assumed that IGF-1 mediating bGH action may increase milk fat production by stimulation ACC with phosphorylation (short term) and/or increasing amounts of the enzyme proteins (long term). Consequently, the main purpose of this paper is to give the readers not only the galactopoietic effects of bGH, but also the insight of bGH action with regard to stimulating milk fat synthesis from the whole body to the molecular levels.

(Key Words: Bovine Growth Hormone, Insulin-Like Growth Factor-1, Milk Fat Synthesis, Acetyl-CoA Carboxylase)

INTRODUCTION

It has been well documented that bGH stimulates milk production via the action of IGF-1, somatomedin (Bauman and McCuacheon, 1986; Johnson and Hart, 1986; Peel and Bauman, 1987; Bauman, 1992; Forsyth, 1996). This classical somatomedin hypothesis of bGH is that bGH triggers the liver to secrete IGF-1, which then transported though blood to act on specific target tissues including mammary glands. We do also believe that bGH performs the dual actions, with which it acts on some tissues to exert catabolic functions, such as stimulating lipolysis in adipose tissues by the direct manner, whereas acts on other tissues to give rise to anabolic functions, such as increasing milk components in mammary glands by the indirect way, using the bGH mediator, IGF-1.

Even though this dogmatic hypothesis has been tremendously studied and modified, it has not been fully elucidated mainly because the actions of IGF-1 are complex, and the proper cell culture system is not established. Two phenomena in the IGF-1 properties may explain the complexity of IGF-1 actions. One is that IGF-1 is performed by endocrine, paracrine and/or autocrine actions, by which the IGF-1 level in serum does not indicate the exact concentration of acting IGF-1 on the

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target tissues. The other is that specific IGF-binding proteins (IGFBPs) exist in serum that modulate IGF-1 actions and its clearance from serum, which makes the researchers to interpret IGF-1 data very difficult. Regardless of the improper elucidation of the mode of bGH action, recombinantly derived bGH (rbGH) has been commercialized throughout the world except the European countries.

Even then, unfortunately, no one have explored the entire possible mechanism of bGH action from the whole body to the cellular and/or molecular levels. Therefore, with the coverage of the galactopoietic effects of bGH at the whole body level, we discussed the possible mode of bGH action via IGF-1 by examining the IGF-1 effects specifically on milk fat synthesis at the body and tissue level as well as acetyl-CoA carboxylase (ACC) at the molecular level.

**BOVINE GROWTH HORMONE (bGH) AND LACTATION IN DAIRY CATTLE**

**Biochemical nature**

Growth hormone (GH) is produced by somatotropes, a subclass of pituitary acidophilic cells in the anterior pituitary glands of animals, and released in a pulsatile manner (Ross and Buchanan, 1990). GH secretion is primarily controlled by a dynamic equilibrium between the inhibitory and stimulatory hypothalamic peptides, somatostatin and GH-releasing factor. Secretion of these hypothalamic peptides and controlled by neuropeptides and neurotransmitters which are, in turn, influenced by GH feedback, age, sex, hormones and nutritional status (Buonomo and Baile, 1990).

bGH has been isolated from the pituitary gland and its amino acid sequences were elucidated as two different amino acid sequences containing either 190 or 191 amino acids (Wallis, 1975; Kostyo and Reagan, 1976). It was also observed that bGH has an allelic variation at position 126, which leads to a 2:1 ratio of leucine to valine (Wood et al., 1989). Therefore, four different bGH are produced by the pituitary gland. bGH contains two intra-chain disulfide bridges and consists mainly of four anti-parallel \( \alpha \)-helices which are arranged in a left-twisted, tightly packed helical bundle (Abdel-Meguid et al., 1987).

In terms of physiological roles of GH, it has been known that GH exerts, either directly or indirectly, anabolic responses such as bone growth, cell division and protein synthesis, increases the oxidation of lipid, and inhibits the transport of glucose into body tissues (Pell and Bates, 1990). A primary physiological role of GH action is assumed to be the preservation of body protein, particularly during periods of energy deficit, by reducing proteolysis and stimulating the incorporation of amino acids into muscle while diverting glucose and fatty acids away from tissue deposition, thus making them available as energy sources (Pell and Bates, 1990).

**Effects on lactation**

Asimov and Krouze (1937) reported for the first time that injections of crude extracts of bovine anterior pituitary glands enhanced milk production in lactating cows. Young (1947) showed that the galactopoietic response was due to bGH in the pituitary extracts. Later, Cowie et al. (1964) demonstrated that bGH was the major galactopoietic hormone was essential to the maintenance of normal lactation in ruminants. In contrast, prolactin plays the role of major galactopoietic hormone in the rat (Sinha et al., 1974).

Among the early studies, Brumby and Hancock (1955) conducted the most comprehensive evaluation of the galactopoietic effect of GH in lactating cows. Daily injections of 50 mg bGH for 12 weeks beginning in the second month of lactation increased milk production by 72% while milk production increased 44% when treatments were initiated the ninth month of lactation. The results of this study were verified when Machlin (1973) administered 90 mg of highly purified bGH to lactating cows three times a week. After an initial dramatic increase in milk production the lactational profiles declined in parallel with those of control cows. Treatment resulted in a sustained increase of 25-30% in milk production over a 10 week period.

Further progress in bGH research was limited until the early 1980s mainly because of shortages and impurities of pituitary gland extracts from slaughtered animals (Johnsson and Hart, 1986). However, recombinant DNA technology finally made large amounts of pure bGH available to the researchers, resulting in stimulating research into re-examinations of the galactopoietic role of bGH and of biological actions of the hormone in the lactating cows (Peel and Bauman, 1987). Taking advantage of the availability of this product, Bauman et al. (1982) conducted the first study with dairy cows using rbGH. They showed that rbGH was as effective as pituitary-derived bGH in enhancing milk production. Since then, a tremendous amount of the research with rbGH (more than 1,000 bGH studies using over 20,000 dairy cows) has been conducted (Bauman, 1992). Milk production has been increased up to 40% with daily injections of rbGH (Peel and Bauman, 1987). Most experiments have utilized rbGH doses between 10 to 50 mg/d with maximum milk response achieved at a rbGH
daily injection of approximately 30-40 mg/d (Johnsson and Hart, 1986; Peel and Bauman, 1987).

Effects on milk fat production

Milk fat is mainly composed of triglycerides which make up 97 to 98% of bovine milk fat (Bauman and Davis, 1974). The fatty acids in milk triglycerides are derived from two sources, circulating lipid in blood and de novo synthesis in mammary secretory cells (Moore and Christie, 1981). It has been experimentally determined that the fatty acids of milk fat with carbon chains of C<sub>4</sub>-C<sub>14</sub> (short- and medium-chain fatty acids) and 50% of C<sub>16</sub> arise from de novo synthesis within the mammary gland. In contrast, long-chain fatty acids such as C<sub>18:1</sub> are derived from circulating lipids and arise from either dietary sources or body fat reserves (Christie, 1981). Approximately 40% by weight or 60 molar % of milk fatty acids are synthesized in the mammary gland (Bauman and Davis, 1974). It has been well documented that administration of either pituitary-derived or recombinant-DNA-derived bGH enhance milk production and milk components, including milk fat in dairy cows (Johnsson and Hart, 1986; Peel and Bauman, 1987). Studies involving both short-(less than 30 d) and long-term (more than 30 d) treatments with bGH or rbGH increase milk fat yields by 10-40% (Peel and Bauman, 1987).

Peel et al. (1981) examined effects of highly-purified bGH on milk fat during early lactation in high-yielding dairy cows. bGH 944 mg/d) was injected to Holstein cows for 11 days beginning on day 74 of lactation. The cows treated with bGH increased milk fat production (22.7%). This increase significantly surpassed the increase in milk production (9.5%). The concentration of NEFA in plasma was remarkably elevated as a result of bGH treatment when cows were in negative energy balance during the experiment period. A similar study conducted by the same group of researchers (Peel et al., 1983) in which bGH (44 mg/d) was administered to lactating Holstein cows in early (wk 12) and late lactation (wk 35) for 10 days. In early lactation, bGH caused an increase of milk fat yield (17%) which was proportional to the increase in milk yield (15%). However, in late lactation, the increase in milk fat yield (42%) by bGH treatment markedly exceeded the increase in milk yield (31%). During control periods, cows were in similar positive energy balance at each stage. However, the reduction in feed intake concurrent with bGH treatments in late lactation resulted in negative energy balances during treatment, whereas during early lactation cows remained in positive energy balance. bGH significantly increased NEFA concentration (68%) of plasma in late lactation, however, only a slight increase in NEFA concentration occurred in early lactation.

Based on the above early studies, researchers have discussed the hypothesis that the energy status of the cow at the time of treatment determines effects of bGH on milk fat. Therefore, bGH increases milk fat yield more than milk yield when cows are in negative energy balance, but proportionally enhances the production of milk and fat, when cows are in positive energy balance. This hypothesis has been further supported by subsequent studies in which bGH markedly enhanced milk fat yields exceeding increases in milk yields when cows were in negative energy balance (Bitman et al., 1984; Eppard et al., 1985; McCutcheon and Bauman, 1986; Lynch et al., 1992), while increased milk fat yields induced by bGH were similar to increase in milk yield when cows were in positive energy balance (Fronk et al., 1983; Bauman et al., 1985, Eppard et al., 1985; Lynch et al., 1992).

Examination of effects of bGH on the fatty acid composition of milk fat provides insight to the mechanism of bGH stimulation of milk fat synthesis. Bitman et al. (1984) reported that short-term administration (14d) of bGH (44 mg/d) to lactating Holstein cows in negative energy balance resulted in less short- and medium-chain fatty acids and more long chain fatty acids, mainly C<sub>18:1</sub>, in milk fat. The increase in milk fat yields from preformed fatty acids (long-chain fatty acids) with bGH treatment was about three times that of those synthesized de novo. These workers also suggested that the enhanced portion of C<sub>18:1</sub> in milk fat possibly were derived from increased mobilization of lipid reserves since diet composition did not change. Another study (Eppard et al., 1985) demonstrated that bGH shifted the fatty acid composition of milk fat to an increase in the percentage of C<sub>18:1</sub>. When bGH was administered at 30.9 mg/d to lactating Holstein cows beginning in 15 wk postpartum and continuing for the duration of the lactation, a decrease in the percentages of short- and medium- chain fatty acids and increase in the percentages of long-chain fatty acids was observed (Baer et al., 1985). Differences in fatty acid compositions between bGH-treated and control cows were greatest during the first 8 wk of bGH administration. These difference became smaller as bGH treatment continued.

The comprehensive study was recently completed by Lynch et al. (1992) with nine Holstein cows administered biweekly with a prolonged-release formulation of rbGH commencing at 9 wk postpartum and continued for the full lactation. Administration of rbGH increased milk fat production by increasing output of both short- and
medium-chain fatty acids (de novo synthesis) and long-chain fatty acids (preformed fatty acids). Although average fatty acid composition of milk fat was not affected by rbGH treatment, during the first 13 wk of treatment the percentage of \( C_{18:1} \) was higher in milk from rbGH-treated cows. The difference gradually declined as energy balance became less negative. Injection of bGH consistently decreased the percentage of \( C_{18} \) in the milk fat. They reported that a possible explanation for a differential effect of bGH on \( C_{18} \) versus \( C_{18:1} \) is that rbGH treatment might enhance the activity of mammary stearoyl-CoA desaturase.

The mode of bGH action underlying effects on milk fat production

At the onset of lactation the mammary gland has metabolic priority over other tissues through coordinated changes, resulting in increasing blood flow to the gland, decreasing the utilization of substrates by peripheral tissues and increasing the metabolic capacity of mammary tissues. This coordination of nutrient partitioning is accomplished by integrated changes in hormone secretion and receptor populations (Bauman and Currie, 1980; Dehoff et al., 1988). In the dairy cow, plasma bGH concentrations increase abruptly at parturition (Ingails et al., 1793; Oda et al., 1989). It has been generally believed that GH acts as a homeorhetic controller that shifts partitioning of nutrients in support of milk synthesis. Therefore, bGH effects are primarily upon directing the availabilities of absorbed nutrients to the mammary gland. This involves coordinating the metabolism of various body organs and tissues (Johnsson and Hart, 1986; Peel and Bauman, 1987; Vernon, 1989).

One of the major effects of bGH on milk fat production seems to be due to change in capacity of adipose tissues. An increase in plasma concentrations of NEFA with bGH administration in vivo and an increase in NEFA release in short-term incubations of bovine adipose tissue in vitro (Kronfeld, 1965; Bines and Hart, 1982) support this suggestion. It seems that effects of bGH on adipose tissue are direct. Hart (1983) reported that goats treated with ovine GH dramatically reduced the degree to which hypoglycemia is induced by an insulin challenge. In vitro studies with bovine and ovine adipose tissue demonstrated that incubation for a few days with GH antagonized the responsiveness of adipose tissue to insulin (Vernon, 1982; Etherton et al., 1987; Marichenko et al., 1992). When bGH was administered to lactating cows, the increase in NEFA following an epinephrine challenge is more than doubled relative to controls (McCUTCheon and Bauman, 1986). Therefore, it was suggested that bGH treatment alters the responsiveness of adipose tissue to hormonal signals, presumably by altering numbers and/or affinities of receptors or degree of enzyme activation (Peel and Bauman, 1987). This hypothesis is supported by the studies demonstrated that an increase in the activity of hormone sensitive lipase and decreases in lipoprotein lipase and acetyl-CoA carboxylase in rats administered GH (Bunyan and Greenbaum, 1965; Goodman, 1963; Yoshio et al., 1981). Although it is known that bovine adipose tissue has bGH receptors (Collier et al., 1984), no clear cellular mechanisms have been elucidated regarding effects of bGH on bovine adipose tissues.

Rates of blood flow are one of the major factors affecting the intracellular availability of substrates (NEFA, acetate and 3-hydroxybutyrate) for milk fat synthesis in bovine mammary glands. Since rates of extraction of many milk precursors are high, there is close correlation between mammary blood flow per unit weight of tissue and milk production (Linzell, 1974). The increase in milk output during GH treatment has been shown to be associated with proportional increases in mammary blood flow in goats (Hart et al., 1980; Meapham et al., 1984) and cows (Davis et al., 1983; McDowell et al., 1984). McDowell et al. (1984) indicated that changes in blood flow contributed more to the increased uptake of glucose, acetate and 3-hydroxybutyrate than changes in plasma concentrations or extraction percentages across the gland. Overall, increases in cardiac output and peripheral blood flow appear to be general actions of GH in both lactating and non-lactating ruminants (Johnsson and Hart, 1986). Cellular mechanisms responsible for these effects of bGH are not clear. It has been assumed that bGH, acting indirectly via IGF-1, increases the metabolic activity of mammary glands resulting in increased O\(_2\) requirements and CO\(_2\) production, resulting in increasing rates of blood flow to the mammary gland (Bauman and McCUTCheon, 1986).

The early hypothesis that the enhanced rate of milk production during bGH injection may be only a passive response to the availabilities of additional nutrients to the mammary gland as a result of GH effects on other tissues was tested by the study of Peel et al. (1982) which demonstrated that milk component yields including fat were not increased, when lactating cows were supplied with additional nutrients postpartum. This study was confirmed by Lough et al. (1984) who reported that there was no increase in milk synthesis when additional nutrients were supplied in combination with bGH as compared to bGH alone. Thus, nutrient availability, expressed in terms of arterial concentrations, may not be
rate-limiting to increases in milk component yields, including fat, during GH administration. In addition, Knight and Peaker (1984) suggested that the decrease in milk yield during the course of a normal lactation is assumed to be due to a decline in the number and/or activity of secretory cells in the mammary gland which occurs in spite of relative improvements in nutrient balance in later lactation. Therefore, one may suggest that bGH may enhance the metabolic capacity of mammary glands to increase milk production due to an increase in the number or activity of epithelial cells of bovine mammary glands.

The mode of action of bGH upon the mammary gland has not been elucidate. Although bGH receptor mRNA has been detected in bovine mammary epithelial cells (Glim et al., 1990), bGH receptors have not been detected on the membranes of epithelial cells in bovine mammary glands (Akers, 1985; Kazmer et al., 1986). In addition, infusion of bGH directly into the mammary artery did not increase milk production in sheep (McDowell and Hart, 1984). Therefore, it appears that bGH does not directly act on mammary glands.

Many of the anabolic effects derived from bGH have been suggested to be mediated by IGF-1 (Pell and Bates, 1990). bGH administration to lactating dairy cows increased the plasma concentrations of IGF-1 (Davis et al., 1984; Peel et al., 1985; Glimm et al., 1988). The presence of high affinity receptors for IGF-1 in bovine mammary glands has been demonstrated (Campbell and Baumrucker, 1986; Deheff et al., 1988; Glimm et al., 1988). Further, lactation is associated with increases in the concentrations of IGF-1 receptors in bovine mammary glands (Dehoff et al., 1988). Thus, it is highly possible that bGH acts on the mammary gland indirectly via its positive effects upon IGF-1 synthesis and secretion by liver.

IGF-1 AS A MEDIATOR OF GH

IGF-1

It has been well known that IGF-1 has both metabolic and mitogenic activities in many cells and tissues (Van Wyk, 1984; Lowe, 1991; Cohick and Clemonns, 1993). IGF-1 contains a number of insulin-like properties, primarily a strong hypoglycemic effect, thus has been named as "insulin-like growth factor" (Ross and Buchanan, 1990). Like insulin, IGF-1 is composed of B and A domains containing 70 amino acids with molecular weights of about 7,500 Da and shows high structural homology to insulin (49%) (Rinderknecht and Humbel, 1978). The amino acid sequences of the mature circulating form of IGF-1 is more than 92% conserved among mammalian species (Foyt and Roberts, 1991). The amino acid sequence of IGF-1 purified from bovine serum is identical to that purified from human serum (Honegger and Humbel, 1986).

While the majority of circulating IGF-1 is of hepatic origin (Froesch et al., 1985), many other tissues contain significant concentrations of IGF-1, as a results of local production (D'Ercole et al., 1984). Thus, synthesis of IGF-1 at a lot of locations suggests that may function in an autocrine/paracrine fashion in addition to having the classical endocrine mode of action. In the bovine, IGF-1 has been also detected in colostrum and milk (Malven et al., 1987; Francis et al., 1988), follicular fluid (Spicer et al., 1988), and mammary lymph (Lacasses et al., 1991).

Relationship between bGH and IGF-1

The observation that cartilage from hypophysectomized rats was unresponsive to GH added in vitro, but incorporated sulphate in response to normal rat serum and serum from GH-treated hypophysectomized rats (Salmon and Daughaday, 1957) led to the original IGF-1 hypothesis. This hypothesis suggested that GH from the pituitary exerts its effects by stimulating IGF-1 release from the liver which then mediates anabolic actions in target tissues.

However, two lines of evidences argued against the original hypothesis. One of these arose from studies demonstrating that many tissues other than liver produce significant levels of IGF-1 (D'Ercole, 1984). This observation has been confirmed by demonstrations of the presence of IGF-1 mRNA in many extrahepatic tissues (Murphy et al., 1987; Han et al., 1988). The other evidence is that GH has direct anabolic actions on some tissues mediated via GH receptors instead of via IGF-1. For example, local administration of GH into the proximal tibial epiphyseal plates of hypophysectomized rats stimulates longitudinal bone growth on the injected side (Russell et al., 1985). Local administration of GH through cannulae implanted into the bony epiphysis produces a consistent, dose-dependent response suggesting that GH effectively interacts with its target cells by this route of administration (Isgaard et al., 1986). In addition, GH stimulates differentiation of pre-chondrocytes in the epiphyseal growth plate which then undergo chonial expansion in response to locally expressed IGF-1, suggesting that GH and IGF-1 are both required for bone growth (Isaksson et al., 1987). Further, IGF-1 mRNA levels has been shown to be regulated by GH in many extrahepatic tissues (Holly and Wass, 1989).

These observations have led to modification of the original IGF-1 hypothesis which defined IGF-1 as the
endocrine mediator of GH action. However, significant amounts of IGF-1 (more than 50% of total body content) are present in the circulation and originated from hepatic production of IGF-1 in rats (Schwander et al., 1983). This together with the observation that IGF-1 receptors are clearly absent from adult liver (Caro et al., 1988) supports the original hypothesis that hepatic IGF-1 is not acting locally within the liver but enters the circulation to perform some endocrine function. The recent observation of Hodgkinson et al. (1991) that n-methionyl IGF-1 administered to lactating sheep was unequally distributed to target tissues suggests that blood borne IGF-1 may conduct specific endocrine functions in selected tissues. In this study, it was also demonstrated that 85% of IGF-1 in mammary epithelial cells is derived from circulating IGF-1. Therefore, the endocrine function of IGF-1 might be prominent in some tissue and, especially, in mammary glands.

Ronge et al. (1988) reported that in dairy cows, IGF-1 concentrations in circulation were low at peak lactation when GH concentrations were concomitantly enhanced. They also demonstrated a negative relationship between blood IGF-1 concentrations and milk yield over the entire duration of lactation. In contrast, it has been well documented that administration of bGH significantly increases the circulating concentrations of IGF-1 in dairy cattle (Davis et al., 1987; Cohnick et al., 1989; Prosser et al., 1989, Cisse et al., 1991). Furthermore, when the distribution pattern of immunoreactive IGF-1 was determined in both normal and bGH-treated lactating bovine mammary tissues, a prominent elevation in immunoreactive IGF-1 were observed in the cytoplasm of mammary epithelial cells of tissue from bGH-treated cows (Glimm et al., 1988).

**Biological actions on mammary glands**

The mechanisms associated with bGH actions in enhancing milk production are not entirely elucidated. It is obvious from the discussion presented in the previous sections that bGH effects may be mediated, at least in part, by IGF-1. Thus, IGF-1 may act directly on the mammary gland to increase synthesis of milk components. The number and metabolic activities of secretory cells of the mammary glands are the major factors determining the ability of a cow to produce milk (Johnsson and Hart, 1986). However, currently little is known concerning the mitogenic and metabolic effects of IGF-1 on bovine mammary secretory cells.

In *in vitro* studies have shown that IGF-1 stimulated \[^{3}H\]-thymidine incorporation into DNA of mammary gland explants from virgin heifers (Shamay et al., 1988) and lactating cows (Baumrucker and Stemberger, 1989). In these studies, it was not clear whether or not increased DNA synthesis induced by IGF-1 resulted in increased secretory cell numbers or larger cells since the mitogenic effect of IGF-1 was evident in all of the heterogeneous cell types in the mammary gland explants. McGrath et al. (1991) demonstrated that IGF-1 enhances cell proliferation in cultures of mammary gland epithelial cells isolated from non-lactating heifers was demonstrated. In addition, IGF-1 stimulates cell proliferation in a human breast cancer cell line (Myal et al., 1984) and the COMMA-D mouse mammary cell line (Riss and Sirbasku, 1987). It is well documented that IGF-1 is capable of exerting mitogenic effects in a variety of cell types of various species (Lowe, 1991). Therefore, the above findings suggest, but do not prove, that IGF-1 acts as a mediator of bGH by increasing secretory cell numbers rather than increasing metabolic capacities of mammary secretory cells.

In contrast, a number of data suggest that IGF-1 exerts metabolic effects on other bovine cell types. When bovine adrenal fascicula cells are treated with IGF-1, corticosterone biosynthesis is increased in a dose-dependent manner. This augmentation of steroidogenesis is apparently due to increases in the activities of several enzymes in the pathway of steroid biosynthesis (Penhoat et al., 1988). Dopamine \(\beta\)-hydroxylase activity in bovine adrenal chromaffin cells is enhanced by IGF-1 regardless of the mitogenic activity of IGF-1 (Wilson, 1991). Moreover, IGF-1 treatment of bovine granulosa cells stimulates the release of oxytocin and increases oxytocin mRNA levels (Holtorf et al., 1989). In bovine pulmonary artery smooth muscle cells, IGF-1 increases the synthesis of an mRNA encoding tropoelastin (Baësch et al., 1989). It has been also well documented that IGF-1 exerts metabolic effects associated with the stimulation of protein and mRNA synthesis in various cell types of many other species (Lowe, 1991).

Some *in vivo* studies support the possible metabolic effects of IGF-1 on the mammary gland. In a study with goats (Fleet et al., 1988), IGF-1 was infused via the public artery directly supplying either the left or right mammary gland. Infusion of IGF-1 for 6 h caused a 30% increase in the rate of milk secretion in the infused gland compared with a 15% increase in the non-infused gland. Increase in milk secretion on the infused side increased within 4 h of infusion. Further, data on effects of IGF-1 upon metabolic activity per secretory cell are, essentially, limited to the data of Middleton et al. (1988; largely unpublished) which indicated, not always statistically significant, increases in RNA, enzyme and metabolic
activities per unit DNA and/or per total mammary glands from bGH as compared to control cows. If IGF-1 causes increases in milk synthesis by direct actions on the mammary gland, one can assume that IGF-1 increases metabolic activities of mammary secretory cells, resulting from enhancing the activities of rate-limiting enzymes in the pathways of milk component synthesis. Available data support this view but are very limited as noted above.

IGFBPs: Modulators of IGF-1 action

Unlike most polypeptide hormones, the majority of IGF-1 in circulation is bound to high affinity, soluble binding proteins (Baxter and Martin, 1988). IGFBPs function in various ways to modify the biological actions of IGF-1, thus making determinations of IGF-1 actions more complicated (Clemmons, 1991; Cohick and Clemmons, 1993; Jones and Clemmons, 1995). IGFBPs have been detected in the plasma as well as the other biological fluids of a variety mammals (Clemmons, 1991) including cows (Ronge and Blum, 1989). Furthermore, it has been demonstrated that media from bovine tissues, cells, and cell lines in culture also contain IGFBPs (Conover, 1990; Campbell et al., 1991; Cohick and Clemmons, 1991; McGrath et al., 1991).

Six forms of IGFBPs in rats and humans, designated as IGFBP-1 through-6, have been cloned and their complete primary structures has been determined (Cohick and Clemmons, 1993). In bovine, IGFBP-2 (Bourner et al., 1992) and -3 (Spratt et al., 1991) have been cloned and sequenced. Although six different IGFBPs have been identified, five forms are detected using ligand blotting methods in sera of several species (Rechler and Nissley, 1990). Bovine serum contains the five forms of IGFBPs which are present in other species (Cohick et al., 1992). Two forms with molecular weights of 43 Kd and 39 Kd have been identified as IGFBP-3, representing the acid-stable subunit of a high molecular weight complex (150 Kd) which binds most of the circulating IGF-1 (Baxter and Martin, 1989). These two forms appear to be different glycosylation variants of the same gene products (Wood et al., 1988). The 34 Kd band present in human and bovine serum corresponds to IGFBP-2 (Upton et al., 1990). A 29 Kd band in human and rat sera has been identified as IGFBP-1 (Hardouin et al., 1989; Murphy et al., 1991) and the 24 Kd band present in sera of most species has been identified as IGFBP-4 (Shimonaka et al., 1989; Shimasaki et al., 1990).

The functions of IGFBPs have not been completely elucidated. It has been proposed that functions of IGFBPs are: 1) to regulate IGF-1 transport in blood; 2) to regulate the amount and rate of IGF-1 transport from the vascular compartment; 3) to direct the transport of IGF-1 to specific tissues and cell types; 4) to control the binding of IGF-1 to its receptors; 5) to regulate the half-life of IGF-1; and, 6) to regulate IGF-1 actions on target cells by inhibiting or potentiating IGF-1 mediated cell growth responses and metabolic actions (Clemmons, 1991; Jones and Clemmons, 1995).

It appears that IGFBPs have a crucial role in the regulation of the bioavailability of IGF-1. The 150 Kd of ternary complex (IGF-1 · IGFBP-3 · acid-labile unit), which is not able to cross the capillary barrier, protects IGF-1 from enzymatic degradation and rapid clearance from the circulation thereby prolonging the serum half-life of IGF-1 in the circulation (Hodgkinson et al., 1987; Binoux et al., 1991). It has been also reported that IGFBPs may modulate IGF-1 actions at the cellular level. Addition of purified IGFBP-1, -2, -3, or -4 to various cells in culture in culture inhibits binding of IGF-1 to cell surfaces and thus decreases IGF-1 bioactivity by sequestering IGF-1 and preventing receptor interaction (Cohick and Clemmons, 1993). The biological effects of purified bovine IGFBP-3 on IGF-1 action in bovine fibroblasts have been investigated. Co-incubation of bovine fibroblasts with IGFBP-3 inhibits IGF-1 actions on aminosobutyril acid uptake and thymidine incorporation, while preincubation with IGFBP-3 is potentiating (Conover et al., 1990).

Administration of bGH to cows elevated IGFBP-3 concentrations in sera (Vicini et al., 1991; Cohick et al., 1992). This effect is likely to be mediated by IGF-1 because direct infusion of IGF-1 into hypophysectomized rats increases IGFBP-3 (Zapf et al., 1989) and IGFBP-3 levels are increased due to overexpression of an IGF-1 transgene in either normal of GH-deficient transgenic mice (Camacho-Hubner et al., 1991). In contrast to the increase observed in IGFBP-3, circulating IGFBP-2 concentrations in cows declined in response to bGH administration (Vicini et al., 1991; Cohick et al., 1992). This suppressive effect may be due to a direct action of bGH since plasma IGFBP-2 levels are elevated approximately threefold in hypophysectomized rats (Orlowski et al., 1990) and infusion of IGF-1 into two human subjects did not raise the levels of IGFBP-2 when these subjects were treated with GH (Zapf et al., 1990). Therefore, one can assume that the reduction of IGFBP-2 and increase in IGFBP-3 and the acid-labile subunit might cause a decrease in the rate of IGF-1 transport from circulation. Indeed, the decrease in IGFBP-2 concentrations in cow plasma observed during bGH treatment was paralleled by a decrease in IGFBP-2 concentrations in afferent mammary lymph, whereas
IGFBP-3 concentrations did not change (Cohick et al., 1992).

Like the production of IGF-1, liver is not the only source of IGFBPs. IGFBPs are secreted by extrahepatic tissues and several types of cultured cells (Clemmons, 1991). Bovine mammary explants from pregnant non-lactating and lactating non-pregnant cows were found to synthesize and secrete IGFBPs including four forms ranging in molecular weight from 29 Kd to 46 Kd (Campbell et al., 1991). Similar forms of IGFBPs were detected in media from bovine mammary epithelial cells grown in vitro on collagen matrices (McGrath et al., 1991).

Syntheses of IGFBPs by various cell types in culture are known to be regulated by hormonal factors including IGF-1 and insulin (Cohick and Clemons, 1993). Bovine mammary epithelial cells cultured in the presence of IGF-1 produced 20-25 ng/ml IGFBP-2 while control cultures secreted approximately 1.0 ng/ml, whereas insulin did not increase IGFBP-2 production (McGrath et al., 1991). On the other hand, Conover (1996) reported that both IGF-1 and insulin increased the production of IGFBP-1, -2, and -4 in a dose-dependent manner and induced IGFBP-3 when bovine fibroblasts were cultured in serum-free media. Moreover, IGFBP-2 synthesis by a bovine kidney epithelial cell line was enhanced by insulin and IGF-1 (Cohick and Clemons, 1991). These findings are consistent with observations in human fibroblasts (Hill et al., 1989) and rat muscle cells (McCusker and Clemons, 1988), in which insulin and IGF-1 enhanced IGFBP production. However, one can not rule out the opposite effects of insulin and IGF-1 on the IGFBP production because of the observation (Clemmons, 1991) that depending on cell type and IGFBP species, IGF-1 and insulin can either enhance or inhibit IGFBP syntheses.

Signal transduction

From the discussion in the previous sections, it appears that the potential involvement of IGF-1 in mediating the galactopoietic effects of bGH may be to increase the metabolic activities of existing secretory cells in the mammary gland, thereby enhancing rates of synthesis of milk components. Metabolic effects of IGF-1 are the result of a chain of events which are initiated by the binding of IGF-1 ligand to the extracellular portion of its receptor. Conformational changes in the receptor structure caused by the ligand binding activate the cytoplasmic tyrosine kinase-containing domain of the molecule, resulting in transduction of the IGF-1 signal through a mode of action which remains obscure (Yarden and Ullrich, 1988).

The receptors for IGF-1 and insulin have similar morphology, consisting of two a subunits and two b subunits that are connected by disulfide bonds to form the functional $\beta - a - a - \beta$ heterotetrameric receptor complex (Rechler and Nissley, 1985). The similarity of the receptors becomes most remarkable in the tyrosine kinase domain which is the functional part of the receptor (Czech, 1989). Further, both receptors bind IGF-1 and insulin, although they bind their specific ligands with 100- to 1000-fold higher affinities than the cross-reacting ligand (Moxham et al., 1989). These similarities and the similar metabolic effects of IGF-1 and insulin made researchers to postulate that the two receptors mediate similar biological responses (Czech, 1989; Werner et al., 1991).

Since both receptors contain intrinsic tyrosine kinase activity, it seems likely that the signalling cascade from these receptors is initiated by tyrosine phosphorylation of substrate proteins and that sequential phosphorylation events may lead to the biological responses of IGF-1 and insulin (Werner, 1991). Indeed, IGF-1 and insulin stimulate phosphorylation of tyrosine residues in a 175 Kd protein (named as insulin receptor substrate-1) in a rat thyroid cell line, possibly by the receptors (Condorelli et al., 1989). A recent study demonstrated that the tyrosine-phosphorylated 175 Kd protein (receptor substrate-1) binds tightly to phosphatidylinositol 3-kinase, thus activating this enzyme (Giorgetti et al., 1993).

Short-term metabolic effects of insulin result from regulation of key enzymes, at least in part, due to enzyme phosphorylation (Brownsey and Denton, 1987). For example, it is generally believed that insulin increases the activity of acetyl-CoA carboxylase (ACC) by phosphorylating serine/threonine residues of the enzyme at a site distinct from the cAMP-dependent protein kinase phosphorylations site (Brownsey and Denton, 1987). Later, Kim (1989) suggested that activation of different serine/threonine kinases by distinct hormones (insulin and IGF-1 vs glucagon and epinephrine) is responsible for phosphorylating separate serine/threonine residues on ACC. This explains the paradox that both activation and inhibition of ACC can occur by phosphorylation of serine/threonine residues.

No information is available about ACC phosphorylation by IGF-1. However, it was reported that epidermal growth factor activates ACC activity by phosphorylating the enzyme on the same phosphorylation sites as does insulin in rat adipocytes (Heystead and Hardie, 1986a). Taken together, it is reasonable to postulate that IGF-1 may be involved in short-term regulation of ACC, the limiting enzyme in the synthetic pathway of milk fatty
acids (see below) by phosphorylating the enzymes as described for insulin.

In terms of long-term metabolic effects, insulin and growth factors increase phosphorylations of serine residues on ribosomal protein S6 by a phosphorylated and activated ribosomal protein S6 kinase (Smith et al., 1979). This may increase rates of translation since phosphorylation prevents dissociation of 40S ribosomal subunits from polysomes (Thomas et al., 1980; Duncan and McConkey, 1982). Ribosomal protein S6 is a component of the eukaryotic 40S ribosomal subunit and likely to be involved in initiation of translation (Terao and Ogata, 1979).

Iozaki and Kohn (1987) reported that when FRTL-5 rat thyroid cells were treated with IGF-1, c-myc and c-fos known as transcriptional factors were induced without increasing cell numbers. During preadipocyte differentiation, IGF-1 also increases a c-fos protein which in turn induces expression of specific genes related to lipid synthesis including glycerol phosphate dehydrogenase (Distel et al., 1987). Meanwhile, Oemar et al. (1991) observed that stimulation of mesangial cells with IGF-1 results in the tyrosyl phosphorylation of a transcriptional factor c-jun and other nuclear proteins. It has been well documented that transcription factor functions are directly regulated by phosphorylation (Hunter and Karin, 1992).

Therefore, it is possible that in the long-term effects IGF-1 are mediated via activation of transcriptional factors by tyrosine or serine/threonine phosphorylations. These then stimulate transcription of genes for the lactogenic enzymes, such as acetyl-CoA carboxylase, in the mammary gland.

**ACETYL-COA CARBOXYLASE (ACC)**

**Biochemical nature**

Acetyl-CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, which is the first step in fatty acid synthesis. It is generally accepted that ACC is the rate-limiting enzyme in fatty acid synthesis (Volpe and Vagelos, 1976; Wakil et al., 1983; Numa and Tanabe, 1984; Kim et al., 1989). The observations that tissue concentrations of malonyl-CoA vary in parallel with rates of fatty acid synthesis and that ACC activity changes in response to the lipogenic needs of the cell reflect the rate-limiting nature of ACC (Kim et al., 1989).

In terms of cow mammary fatty acid synthesis, two original studies demonstrated the regulatory role of ACC. Mellenberger et al. (1973) reported a correlation of 0.97 between ACC activity and lipogenic capacity in tissue biopsies obtained at four time points from 30 days prepartum to 40 days postpartum in cows. Activities of other bovine mammary enzymes including fatty acid synthetase did not correlate (Baldwin, 1966; Mellenberger et al., 1973).

In general, ACC in homogenized tissue samples is found in the cytoplasm following subcellular fractionation (Volpe and Vagelos, 1976). However, in subcellular fraction studies of rabbit mammary tissue, it was demonstrated that ACC was present in both the cytosol and microsomal fraction (Easter and Dils, 1968). Thus, in vivo, ACC may be loosely associated with the microsomal particles. Aslo, it was found that that liver contains substantial quantities of a relatively inactive but immunologically precipitable form of ACC bound to the mitochondrial outer membrane (Allred and Roman-Lopez, 1988). Roman-Lopez et al. (1989) suggested that the concentration of the active form of the cytosolic ACC increased at the expense of mitochondrial forms when fasted rats were refeed.

In ACC preparations from various animal tissues, analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has shown that a molecular weight of ACC ranges from 220 Kd to 260 Kd (Numa and Tanabe, 1984). Some of this variability may be explained by the use of different molecular weight standards; by the enzyme from different animals having slightly different molecular weights; or by proteolytic cleavage artifacts (Brownsey and Denton, 1987). The complete amino acid sequence of ACC from rat mammary glands was deduced by cloning and sequence analysis of mRNA complementary to its mRNA (Lopez-Casillas et al., 1988). The complete coding sequence containing 7035 bases encodes a polypeptide chain of 2345 amino acids having a molecular weight of about 265 Kd. Recently, a unique biotin-containing cytosolic protein of a molecular weight 280 Kd that is distinct from the 265 Kd form has been identified in rat mammary glands and is considered an isozymic form of ACC (Bianchi et al., 1990).

There is general agreement that ACC exists in at least two major forms which are an active polymer and an inactive protomer (Numa and Tanabe, 1984; Kim et al., 1989). The inactive protomer (dimer) form has a molecular weight of 500 Kd containing two identical subunits (Beaty and Lane, 1983). Each subunit contains one biotin and active sites for the enzyme action. The polymerized active form represents a large filamentous polymer with a molecular weight of up to 10,000 Kd which corresponds to the aggregation of up to 20 inactive dimers to give filaments of up to 0.5 mm in length.
(Brownsey and Denton, 1987). Dissociation to the protomeric form is favored by low protein concentration, NaCl, pH greater than 7.5, palmityl-CoA, and malonyl-CoA, whereas the equilibrium toward the aggregated polymeric form is favored by citrate, acetyl-CoA, high protein concentration, and pH 6.5-7.0 (Lane et al., 1974).

Enzyme preparations are routinely preincubated with 10-20 mM citrate at temperatures between 25°C and 30°C for about 30 min to establish full activation of ACC since no activity of the enzyme is detected without citrate (Lane et al., 1974). Citrate is one of the notable metabolites which mediate the polymerization of ACC. Thus, it has been postulated that citrate functions as a positive activator by inducing polymerization of the enzyme (Kim et al., 1989). This hypothesis has been questioned by the observations that the citrate concentration in the cytosol is an order of magnitude lower than that required for ACC activation in vitro and that there is no apparent correlation between lipogenesis and citrate concentration (Kim et al., 1989).

**Reaction mechanisms**

In the reactions of ACC, biotin bound to the protein becomes carboxylated in a reaction that requires ATP and bicarbonate. In the second step, the carboxyl group of carboxybiotin is transferred to an acceptor, usually acetyl-CoA forming malonyl-CoA (Knowles, 1989). These reactions can be summarized:

\[
\begin{align*}
\text{Biotin-Enz. + ATP + HCO}_3^- & \rightarrow \text{COO--Biotin-Enzyme + ADP + Pi} \quad (1) \\
\text{COO--Biotin-Enz. + Acetyl-CoA} & \rightarrow \text{Malonyl-CoA + Biotin-Enzyme} \quad (2) \\
\text{Overall: Acetyl-CoA + ATP + HCO}_3^- & \rightarrow \text{Malonyl-CoA + ADP + Pi} \quad (3)
\end{align*}
\]

The reaction mechanism of ACC has been relatively well elucidated in Escherichia coli. ACC in E. coli is readily separated into three active subunits: a biotin-carboxyl carrier protein (44 Kd), a biotin carboxylase (98 Kd), and a carboxyltransferase (130 Kd) (Volpe and Vagelos, 1976). The first reaction (1) involves the carboxylation of biotin. The biotin prosthetic group is attached by linkage of its valeric acid side chain to a lysine residue of a biotin-carboxyl carrier protein and plays an essential role in the carboxylation reaction (Alberts and Vagelos, 1972). Carboxylation of the biotinyl moiety of the biotin-carboxyl carrier protein by bicarbonate is catalyzed by the ATP-dependent biotin carboxylase. It has been proven that the carboxylated site of biotin is the l-nitrogen of the ureido ring (Volpe and Vagelos, 1976). The most favored possible mechanism of ATP-dependent biotin carboxylase involves the activation of bicarbonate by ATP to produce carboxyphosphate, then this reactive carboxyphosphate carboxylates the l'-nitrogen of biotin (Knowles, 1989).

In the second reaction (2), carboxyltransferase catalyzes the transfer of the activated carboxyl group from carboxybiotin to acetyl-CoA to form malonyl-CoA (Alberts and Vagelos, 1972). Prior to the transfer of the active carboxyl group to acetyl-CoA, the carboxybiotin must be shifted to the active site of carboxyltransferase (Numa and Tanabe, 1984). The swinging arm theory accounting for this movement is that the length, rotation and flexibility of the link between biotin and its carrier protein allow the activated carboxyl group to move from one active site to another (Numa and Tanabe, 1984).

In contrast, the eukaryotic ACC is composed of one kind of polypeptide which carries out the functions of the biotin-carboxyl carrier protein, biotin carboxylase, and carboxyltransferase as well as the regulatory function, thus exhibiting a single, integrated multifunctional polypeptide (Wakil et al., 1983). Recently, the cDNA coding for ACC in the rat mammary gland was cloned and sequenced (Lopez-Casillas et al., 1988). On the basis of this sequence identity and availabilities of cDNAs for the other biotin-containing enzymes, the functional domains of ACC have been tentatively identified. The functional domains of the ATP and HCO3-interaction site, and acetyl-CoA and biotin binding sites have been proposed in the deduced amino acid sequence of rat mammary ACC (Lopez-casillas, 1988). Two proline residues at positions away from the biotin-binding site could serve as hinges for bending the protein so that the ATP and HCO3-interaction site comes close to the biotin site, thus possibly allowing a biotin-containing arm to flip-flop between the carboxyl donor and acceptor sites (Kim et al., 1989).

**HORMONAL REGULATION OF ACC**

**Short-term**

Short-term hormonal regulation of ACC is achieved by modulating enzyme activity covalently resulting from phosphorylation and dephosphorylation of the enzyme by kinases and phosphatases, respectively (Kim, 1983; Brownsey and Denton, 1987). These effects can be observed within a few minutes of exposure of cells to hormones and thus are very unlikely to involve changes in amount of enzyme (Kim, 1983; Brownsey and Denton, 1987). Incubation of rat adipocytes with 32P phosphate results in the incorporation of 32P into ACC (Brownsey et
al., 1977). Carefully prepared ACC from both rat liver and mammary glands contains multiple phosphates which are as many as 8 moles of phosphate per mole of ACC (Witters and Vogt, 1981; Thampy and Wakil, 1985). These observations indicate that ACC in animal tissues is intrinsically phosphorylated.

In rat mammary glands, inhibition of fatty acid synthesis by cyclic AMP (cAMP)-elevating hormones such as glucagon and epinephrine has not been demonstrated (Pluciński and Baldwin, 1982, Clegg et al., 1986). The mammary gland is refractory to the usual events initiated by an increase in intracellular cAMP (Clegg et al., 1987) and glucagon receptors are absent from rat mammary epithelial cells (Robson et al., 1984). In contrast, it is well established that glucagon induced increases in cAMP inhibits fatty acid synthesis in rat hepatocytes (Witters et al., 1979; Holland et al., 1984). In adipocytes, epinephrine (Brownsey et al., 1979; Zammit and Corstorphine, 1982) and glucagon (Zammit and Corstorphine, 1982) have similar effects.

Lee and Kim (1979) preincubated rat adipocytes with $^{32}$P phosphates for 3 hr and then examined the effects of epinephrine on both phosphorylation and ACC activity after 30 minute more incubation. During the 30 minute period no further incorporation of $^{32}$P into ACC of the control tissue observed. In contrast, in the presence of epinephrine, the incorporations of $^{32}$P into ACC almost doubled within 30 minutes while the enzyme was inactivated by 61%. Witters et al. (1979) also provided evidence of a relationship between phosphorylation and inactivation of rat hepatocyte ACC following glucagon treatment. Subsequently, similar observations that glucagon and epinephrine stimulate phosphorylation of ACC and decrease enzyme activity have been documented in rat hepatocytes by glucagon (Holland et al., 1984; Mabrouk et al., 1990) and epinephrine (Mabrouk et al., 1990), and in adipocytes by epinephrine (Witters et al., 1983; Haystead et al., 1990) and glucagon (Holland et al., 1985).

Rat liver ACC which has been phosphorylated and inactivated with a protein kinase can be dephosphorylated and reactivated by incubating with a protein phosphatase (Carlson and Kim, 1973). This finding was supported by the subsequent observations that incubating purified ACCs from lactating rat and rabbit mammary glands with protein phosphatases resulted in dephosphorylation and activation of ACC (Hardie and Cohen, 1979; Hardie and Guy, 1980).

The physiological significance of the relationship of phosphorylation status and ACC activity has been investigated. Citrate is a required activator of animal ACC with half maximal activation observed at concentrations of 2-10 mM (Kim, 1979). This citrate concentration is significantly higher than intracellular concentration of 0.15-0.45 mM and thus may not be an appropriate indicator of the rate of fatty acid synthesis (Kim, 1983). Holland et al. (1984) demonstrated that glucagon causes a significant decrease in fatty acid synthesis by phosphorylation of ACC in rat hepatocytes without a concomitant decrease in the cellular content of citrate. Some studies demonstrated that the dephosphorylated ACC requires less citrate for maximum activation than the phosphorylated forms (Carlson and Kim, 1974; Hardie and Guy, 1980). In detail, phosphorylated ACC has a Km for citrate of 2.4 mM, whereas the Km of the active dephosphorylated form is 0.2 mM (Carlson and Kim, 1974).

Further, the phosphorylated forms of ACC are more susceptible to inactivation and inhibition by various negative effectors such as palmitoyl-CoA, avidin and ATP than the dephosphorylated forms (Carlson and Kim, 1974; Brownsey et al., 1979). Taken together, these observations suggest that covalent phosphorylation of ACC is prerequisite for the functions of the allosteric mechanisms in the presence of metabolites and support the hypothesis that changes in the phosphorylation status of ACC can influence activity at physiological concentrations of citrate (Kim et al., 1989).

In addition, it has been observed that phosphorylation and inactivation of ACC are accompanied by the conversion of active polymers into inactive protomers (Lent et al., 1978; Shiao et al., 1981), whereas dephosphorylation of ACC by a phosphatase results in polymerization of ACC (Thampy and Wakil, 1988). Mabrouk et al. (1990) showed that there are more inactive protomer forms of ACC in the livers of rats injected glucagon or epinephrine and that these protomers have a higher phosphate content than protomers isolated from the livers of control animals.

The signal transduction system responsible for phosphorylation of ACC by cAMP-elevating hormones has been intensively investigated. Acetyl-CoA carboxylases purified from rat mammary glands (Hadie and Cohen, 1978; Hadie and Guy, 1980; Munday and Hardie, 1984) and rat livers (Tipper and Witters, 1982; Witters et al., 1983) are phosphorylated and inactivated by cAMP-dependent protein kinases. These observations are consistent with those found after exposure of intact adipocytes and hepatocytes to epinephrine and glucagon as described previously. Mapping of tryptic or chymotryptic $^{32}$P-peptides isolated by high performance liquid chromatography (HPLC) revealed that the major $^{32}$P-
peptide phosphorylated by epinephrine in rat adipocytes (Brownsey and Hardie, 1980; Witters et al., 1983; Holland et al., 1985) co-migrated with the major 32P-peptide phosphorylated by cAMP-dependent protein kinase. These findings suggest that cAMP-elevating hormones inactivate ACC via activation of the cAMP-dependent protein kinase.

Three cAMP-independent ACC kinases which completely lack sensitivity to protein inhibitors of cAMP-dependent protein kinases were isolated from lactating mammary glands (Munday and Hardie, 1984). Among these kinases, ACC kinase 3 is known to cause inactivation of ACC (Munday et al., 1988b). This enzyme is stimulated by phosphorylation and AMP (Carling et al., 1989) and is now termed AMP-activated protein kinase (Hardie et al., 1989). Recent studies (Munday et al., 1988a; Davis et al., 1990) demonstrated that cAMP-dependent protein kinase which phosphorylates at ser-77 and ser-1200 caused modest inactivation while AMP-activated protein kinase which phosphorylates at ser-79 and ser-1200 causes a much more dramatic inactivation in ACC purified from lactating rat mammary glands. Phosphorylation of ser-77 and ser-79 was responsible for the observed inhibition of the activity (Munday et al., 1988a; Davis et al., 1990). Further, when ACC was isolated from rat hepatocytes and adipocytes which had been incubated with glucagon and epinephrine and 32P-peptide containing ser-77 and ser-79 was sequenced, Ser-79 was phosphorylated but ser-77 was not (Sim and Hardie, 1988; Hastyead et al., 1990). This lack of phosphorylation of ser-77 demonstrated that although cAMP-dependent protein kinase phosphorylates ACC in cell-free extracts, this phosphorylation does not occur in intact cells (Hastyead et al., 1990). This observation strongly suggests that ACC may not be a physiological substrate for cAMP-dependent protein kinase, and that cAMP-elevating hormones may cause phosphorylation and inactivation of ACC through AMP-activated protein kinase, which is, in turn, activated by a 'kinase kinase' system (Hastyead et al., 1990).

On the other hand, it is well established that insulin stimulates fatty acid synthesis by increasing ACC activity within a few minutes in rat adipocytes (Brownsey and Denton, 1982; Zammit and Corstorphine, 1982), hepatocytes (Witters et al., 1979; Buechler et al., 1984), and lactating mammary acini (Williamson et al., 1983). In ruminants, studies of insulin responses of the lipogenic tissues have been greatly hampered by the generally low and variable response of the tissue to insulin during short-term (up to 3 hr) incubations (Vernon and Sasaki, 1989). However, Yang and Baldwin (1973) demonstrated that insulin caused a doubling of rates of fatty acid synthesis in bovine adipocytes during the short-term incubations. Although lactating bovine mammary glands appear to be refractory to insulin effects on fatty acid synthesis (Laarveld et al., 1985), insulin is able to bind to bovine mammary glands due to the presence of insulin receptors (Oscar et al., 1986).

Although insulin and cAMP-elevating hormones have contrasting effects on catalytic activity of ACC and fatty acid synthesis, insulin activation of ACC is also associated with an increase in the overall extent of phosphorylation of the enzyme in rat hepatocytes (Witters, 1981; Holland and Hardie, 1985) and adipocytes (Brownsey et al., 1977; Brownsey and Denton, 1982; Hastyead and Hardie, 1986: Hastyead and Hardie, 1988).

However, two-dimensional analyses of tryptic peptides derived from 32P-labelled ACC isolated from rat adipocytes by immunoprecipitation demonstrated that the principle sites exhibiting increased phosphorylation in the adipocytes exposed to insulin was quite distinct from those in cells exposed to epinephrine (Brownsey and Denton, 1982). The major effect of insulin was to cause a marked increase in the phosphorylation of a peptide (I-peptide), whereas epinephrine resulted in increased phosphorylation of a separate group of two to three peptides with similar mobilities (Brownsey and Denton, 1982). Similar results were obtained by Witters et al. (1983) and Holland and Hardie (1985) using rat hepatocytes and adipocytes, respectively. Further, the polymerized form of ACC, which is active in the absence of citrate, was found to exhibit increased phosphorylation within the I'-peptide in rat adipocytes (Borthwick et al., 1987). Taken together, these studies demonstrated that exposure of rat adipocytes to insulin and epinephrine led essentially to phosphorylation of different sites on ACC and suggesting that these different patterns of phosphorylation by different protein kinases might be associated with opposite changes in enzyme activity.

It has been in dispute whether the increased phosphorylation by insulin causes activation of the enzyme since the effect of insulin on the enzyme activity does not persist during enzyme purification, although the phosphorylation state is preserved (Brownsey and Denton, 1982; Hastyead and Hardie, 1986b; Witters et al., 1988). This contrasts with the inactivating effects of cAMP-elevating hormones, which are quite stable to purification (Holland et al., 1984 and 1985). Further, it was reported that insulin activation of ACC apparently involves the phosphorylation of low-molecular-weight effector and not ACC itself in rat adipocytes (Hastyead and Hardie, 1986b).
Recently, Mabrouk et al. (1990) reported that ACC from livers of untreated rats was relatively low in activity and high in phosphate content. Whereas the enzyme from livers from rats that received insulin has higher activity and lower phosphate content. Thus, this study suggests that insulin causes activation of the hepatic ACC by dephosphorylation of ACC.

Borthwick et al., (1990) first showed that parallel increases in phosphorylation and activity of ACC occur in rat adipocytes exposed to insulin. In this study, the co-purification of insulin-sensitive ACC kinase with the low-molecular-weight component allowed demonstration of parallel increase in phosphorylation and activity of ACC under conditions where phosphorylation of the ACC subunit appears to be occurring primarily in serine residues of the 'T'-peptide. Therefore, it was suggested that the insulin-sensitive ACC kinase apparently requires the presence of low-molecular-weight component which might have been removed during conventional purification techniques (Borthwick et al., 1990).

It has also been reported that epidermal growth factor stimulates both fatty acid synthesis and phosphorylation of ACC in rat hepatocytes (Holland and Hardie, 1985) and adipocytes (Haystead and Hardie, 1986a) within a few minutes. Further, Holland and Hardie (1985) demonstrated that epidermal growth factor and insulin showed the same phosphorylation patterns in ACC when tryp tic digests were analyzed by reverse-phase HPLC. These observations imply that other growth factors including IGF-1 may stimulate fatty acid synthesis by phosphorylation and activation of ACC in major lipogenic tissues including lactating mammary glands. This seems reasonable since the plasma-membrane receptor for insulin is known to share many similarities with receptors for growth factors, including amino acid sequence homologies and the cytoplasmic regions of both receptors contain a tyrosine kinase which is activated by binding of insulin or growth factors (Ullrich et al., 1985).

**Long-term**

In addition to the short-term hormonal regulation of ACC by changes in the catalytic efficiency per enzyme molecule brought by phosphorylation/dephosphorylation, the long-term hormonal regulation of ACC by changes in the number of enzyme molecules plays an essential role in the control of ACC (Numa and Tanabe, 1984). It has been well documented that ACC activity of a variety of animal tissues undergoes long-term regulatory changes in accord with the rate of fatty acid synthesis under a variety of nutritional, hormonal, genetic and development conditions (Numa and Yamashita, 1974; Volpe and Vagelos, 1976; Numa and Tanabe, 1984). The changes in ACC activity that occur over a several-hour to several-day period of time have been shown to result from a change in the amount of enzyme protein (Lakshmanan et al., 1975; Kitajima, 1975). Thus, long-term hormonal regulation of ACC involves changes in the amount of ACC through modification of rates of synthesis and/or degradation of the enzyme protein (Porter and Swenson, 1983). In contrast to the abundance information on the short-term hormonal regulation of ACC, our knowledge of its long-term hormonal regulation is limited.

In ruminants, the mammary gland and adipose tissue appears refractory to short-term effects of insulin on fatty acid synthesis (Vernon and Sasaki, 1989). However, Baldwin et al. (1972) reported that chronic insulin administration to lactating cow significantly increased fatty acid synthesis in mammary glands. When incubations of bovine adipocytes are continued for 24 hr or longer, insulin causes a highly reproducible increases in fatty acid synthesis (Vernon et al., 1985; Etherton and Evock, 1986) and ACC activity (Vernon and Flint, 1989).

Jindal and Pandey (1988) demonstrated that insulin, in combination with prolactin and cortisol, increases ACC activity due to the increased synthesis of the enzyme in explants of goat mammary tissue incubated for 36 hr in the media containing insulin. Recent studies indicate that prolactin also increases rate of fatty acid synthesis in mammary explants from pregnant sheep, concomitant with the increases in activity and amount of ACC when the explants are cultured for 48 hr (Barber et al., 1990 and 1991). In addition, GH exerts its effects on lipogenesis in adipose tissue from lactating sheep by reducing ACC activity, together with a decrease in the amount of enzyme (Vernon et al., 1988).

Unlike ruminants, nonruminants, especially rodents, have been extensively utilized to elucidate the long-term hormonal regulation of ACC. A role for insulin in the long term regulation of ACC is suggested by the observation of markedly decreased hepatic ACC activity in alloxan-induced diabetic rats (Nakanishi and Numa, 1970). In this study, the rate the enzyme synthesis was measured by injecting animals with a dose of $[^{3}H]$ leucine and determining isotope incorporation into enzyme which was precipitated using a specific antibody. Rates of degradation were measured by following loss of isotope from prelabelled enzyme. This study demonstrated that the decrease in enzyme content in diabetic rats was due solely to a corresponding change in the rate of synthesis of the enzyme. Recovery of ACC activities and amounts in liver or adipose tissue occurred with insulin replacement therapy (Volpe and Vagelos, 1974; Pape et
al., 1988; Katsurada et al., 1990).

It was demonstrated that glucagon prevents the marked increase in hepatic ACC activity that results when fasted rats were fed a fat-free diet (Volpe and Marasa, 1975). Rats made thyrotrophic by 7-9 days of injection of triiodothyronine (T3) exhibit approximately twofold higher ACC activities in liver and adipose tissue (Diamant et al., 1972). T3-mediated stimulation (24 to 72 h following T3 treatment) of hepatic ACC in rats was inhibited by cycloheximide and actinomycin D (Das, 1980). In addition, hypophysectomized rats showed a marked reduction in hepatic ACC activity (Volpe and Marasa, 1975).

In an attempt to gain further insight into the molecular mechanism underlying the regulation of synthesis of ACC, Nakaniishi et al. (1976) estimated the relative content of polysomes involved in the synthesis of ACC in the livers of rats subjected to alloxan-diabetes with or without insulin treatment by determining the binding of 125I-labelled antibody to isolated liver polysomes. This study demonstrated that the synthesis of ACC and the amount of ACC bound to liver polysomes were significantly higher in diabetic rats treated with insulin than in untreated diabetic rats, suggesting that increased synthesis of ACC in insulin during a long-term period could be due to changes in the amount of mRNA coding for the enzyme.

The complex physiological interactions imposed on cells in vivo make it difficult to assess the direct role of hormones in the long-term regulation of ACC, thus cell culture provides a useful system for studying the regulation of ACC under defined conditions (Dakshinamuri and Chauhan, 1988). Using rat hepatocyte culture, Kaze and Ick (1981) demonstrated that ACC activity was enhanced approximately two-fold between 24 hr and 48 hr in culture in the presence of insulin. The increase in ACC activity by insulin was blocked by either coryzepin or cycloheximide, suggesting that the activity enhancements might be due to the increased enzyme synthesis. A similar observation was reported by Spence and Pitot (1982). A subsequent study demonstrated that insulin-dependent enhancement of ACC activity in long-term (48 hr) primary culture of rat hepatocytes was due to an increased amount of enzyme (Giffhorn and Kaze, 1984). In this study, incorporation of [35S] methionine and immunoprecipitation of the enzyme revealed that the increase in ACC content was due to an increased rate of enzyme synthesis rather than decreased rate of enzyme degradation. Further, this insulin-dependent induction of ACC was prevented by addition of aminothiol, implying that insulin may regulate ACC activity at the transcriptional level.

Fisher and Goodridge (1978) showed that insulin causes a twofold increase in the relative synthesis of ACC and addition of glucagon plus insulin to the cell culture media abolished the insulin-mediated increase in relative synthesis of ACC. Shafrir and Bieman (1981) demonstrated that human skin fibroblasts grown in lipopoor medium showed a twofold increase in ACC activity upon addition of insulin, and that this increase was abolished in the presence of cycloheximide. Dexamethasone had a permissive effect on insulin induction of ACC in primary cultures of rat hepatocytes (Salati and Clark, 1986). On the other hand, addition of glucagon to Hela cell culture medium caused a 50% decrease in ACC activity accompanied by a corresponding decrease in the relative synthesis of ACC as measured using immunological techniques (Bhullar and Dakshinamuri, 1985).

Collectively, these studies indicate that regulation of the amount of ACC during certain hormonal conditions can be accounted for regulation of the rate of enzyme synthesis. However, the relationship between changes in RNA metabolism and control of enzyme synthesis was not demonstrated in these studies. Recently, the cloning of cDNAs for ACCs allowed researchers to investigate ACC gene activation by hormones through measurement of the abundance of ACC mRNA.

Paper et al. (1988) reported that the amount of ACC mRNA decreased markedly in epididymal fat tissue of diabetic rats as compared to nondiabetic rats, however the mRNA level returned to that of the nondiabetic rats 6 hr after insulin injections. This study also showed that the mRNA of ACC corresponded to changes in the activity and amount of the enzyme. In livers from diabetic rats the transcription rate, mRNA concentration and enzyme induction of ACC markedly decreased as compared to the normal level but, with insulin injection, were increased to the normal level (Katsurada et al., 1990). In this study, the insulin-dependent increase in transcription rate and mRNA concentration of ACC were abolished by cycloheximide treatment, suggesting that de novo synthesis of protein is responsible for the mRNA induction of the ACC gene since relative concentration of B-actin mRNA was not significantly altered by insulin or cycloheximide treatment. This result is in line with the observations that insulin and growth factors, including IGF-1, are able to induce transcriptional factors in some cell line cultures (Stumpo and Blackshear, 1986; Isosaki and Kohn, 1987).

Recently, rat hepatocytes in primary culture were utilized for further studies on the mechanism regulating
the gene expression of ACC by insulin (Fukuda et al., 1992). When the effects of insulin on the mRNA level of ACC were examined in rat hepatocytes during the process of induction, the addition of insulin to culture media markedly enhanced the concentration of ACC mRNA in 16 hr (Fukuda et al., 1992). This study also demonstrated that induction of ACC mRNA was the most sensitive to insulin among the lipogenic enzymes. Further, Foulfelle et al. (1992) reported that insulin stimulates ACC gene expression, resulting in enhanced concentrations of ACC mRNA in cultured rat adipocytes.

**CONCLUSION**

After reviewing the previous studies, we may conclude that bGH stimulates milk production in diverse ways not by disturbing the homeostasis. In terms of the stimulatory effects of IGF-I mediating the bGH action on milk fat synthesis, we postulated that for the short term IGF-I may increase ACC activity by phosphorylating the enzyme, and for the long term it may increase ACC activity by enhancing the enzyme amount resulting from activation of transcriptional factors by tyrosine or serine/threonine phosphorylation followed by stimulating transcription of ACC genes, in bovine mammary secretory cells. We hope that this scenario must be elucidated in the very near future. In order to do that, we must put our energy primarily to overcome the current dilemma, such as, the proper establishment of primary culture system of bovine mammary secretory cells, more detail information about the importance of paracrine and/or autocrine functions of IGF-I as well as IGFBPs, and implications of other signalling molecules/effectors (hormones, growth factors) in the actions of bGH and IGF-I.

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