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BETA-ADRENERGIC RECEPTOR INVOLVEMENT IN LIPOLYSIS OF DAIRY CATTLE SUBCUTANEOUS ADIPOSE TISSUE

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BETA-ADRENERGIC RECEPTOR INVOLVEMENT IN LIPOLYSIS OF DAIRY CATTLE SUBCUTANEOUS ADIPOSE TISSUE

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I hereby recommend that this dissertation prepared under my direction by Edwin Howard Jaster

entitled Beta-adrenergic receptor involvement in lipolysis of dairy cattle subcutaneous adipose tissue

be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Thomas N. Wegner — Dissertation Director — 11/26/79 — Date

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

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Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.

11/78

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ABSTRACT

The influence of lactational state (30 days prior to calving and 30 days postpartum) on adipocyte beta-adrenergic receptors was studied using the binding of (-) [^{3}H] dihydroalprenolol to adipocytes from eight Holstein cows. Lactational state failed ($p > .05$) to modify binding kinetics with similar affinity rate constants, K_1, for dry and lactating cows (8.2 vs. 7.2 X $10^7 min^{-1} M^{-1}$). Estimates of the equilibrium dissociation constant, Kd, of these receptor sites were not affected ($p > .05$) during dry or lactating periods (7.1 vs. 7.9 nM). In contrast, the apparent number of beta-adrenergic receptors varied ($p < .05$) with lactational state (42,154 vs. 72,264 sites/cell), dry and lactating cows respectively, in assays containing 5 nM (-) [^{3}H] dihydroalprenolol.

Glycerol release and adipocyte concentrations of cyclic-AMP were followed during incubation of dairy cow adipocytes with or without 10 μM epinephrine. Epinephrine elicited greater release ($p < .01$) of glycerol in lactating than dry cows (3.91 vs. 2.01 $\mu M/10^6$ cells/120min). In the presence of 10 μM epinephrine, the concentration of adipocyte cyclic-AMP rose during the first 5 min of incubation and then fell. Maximum concentrations of cyclic-AMP at 5 min were not different ($p > .05$) between dry and lactating cows (250 vs. 280 $pM/10^6$ cells).

CHAPTER 1

INTRODUCTION

Adipose tissue in the dairy cow performs two key metabolic functions, the synthesis and the removal (lipolysis) of lipids. Lipolysis can be defined as the hydrolysis of triglycerides to free fatty acids and glycerol in the adipocyte. This key metabolic process of supplying free fatty acids and glycerol is important during periods of caloric insufficiency and stress.

Stress in the dairy cow is best demonstrated by the net energy balance of a high producing dairy cow through a lactation. The high producer has an increased need for the required nutrients and energy during the first 12 weeks of lactation. Even with diligent efforts to maximize feed energy intake, most high producing dairy cows will not consume sufficient ration to supply the energy required for both maintenance and milk production. Early lactation requires the mobilization of body energy stores to meet the stress of negative caloric intake.

The rate of fat mobilization is not constant, but varies in response to physiological alterations. Early lactation is denoted by loss of body fat while late lactation and the early dry period finds the dairy cow in positive energy balance, storing energy as lipid for use during the next lactation (10).

Rate of fat mobilization is also under control of the endocrine system. However, endocrine control of lipolysis in the dairy cow has

received limited examination and the mechanisms involved are not completely understood. One group of hormones known to cause lipolysis in ruminants are the catecholamines (29). In nonruminants, catecholamines appear first to interact with the adipocyte plasma membrane and bind to an adrenergic specific receptor protein. The receptor-adrenergic hormone initiates an intracellular enzyme effect with a sequence of steps that result in the physiological lipolytic response (110).

The relationship of the physiological and pathological states in regard to endocrine receptor involvement in the dairy cow is of considerable interest. It is now apparent that adipose tissue plays a central role in ruminant metabolic disorders, such as ketosis, low milk fat syndrome and "fat cow syndrome". If additional knowledge and understanding of the bovine adipocyte receptor regulation and mobilization of adipose tissue depots was known, this would be of great significance in future attempts to treat metabolic disorders and negative caloric intakes of early lactation.

Objectives of the present study in dairy cows were: 1) identification and evaluation of dairy cow adipocyte beta-adrenergic receptor sites; 2) verify that lipolysis in dairy cow involves the cyclic adenosine 3', 5'-monophosphate (cyclic-AMP) mechanism; and 3) establish lipolytic activity of adipose tissue in dairy cows during different physiological states.

CHAPTER 2

LITERATURE REVIEW

Cyclic-AMP and Lipolysis

Lipolysis, in all animals studied to date, involves the cyclic-AMP phosphorylated protein system. The following is a review of this system.

Studies of the mechanism of action of protein and catecholamine hormones in mammals led to the discovery of a cyclic adenine ribonucleotide in animal tissues (98,99,118). This cyclic nucleotide was identified as cyclic adenosine 3',5'-monophosphate (cyclic-AMP). Researchers working with rat epididymal fat pads (17) were the first to implicate cyclic-AMP as a mediator in lipolytic response of fat with epinephrine.

Cyclic-AMP serves as a key intracellular mediator for the effects of many hormones and other substances that modify cellular function. The mechanism by which epinephrine stimulates lipolysis and levels of cyclic-AMP at any given period in the cell depends upon the activity of two enzymes, adenyl cyclase (119) and low-Km phosphodiesterase (adenosine 3',5'-monophosphate phosphodiesterase)(18,65). Both enzymes synthesize and hydrolyze cyclic-AMP respectively appear to be cell membrane bound enzymes. Cyclase is believed to be located in the plasma membrane. Researchers have substantiated that epinephrine elevates cyclic-AMP levels by stimulation of adenyl cyclase (101). Insulin has been shown to inhibit adenyl cyclase (45,51) although some

researchers have been unable to reproduce this effect, and also stimulate phosphodiesterase (77,81,104,105,132).

Information concerning the mechanism of interaction between hormone receptors and membrane enzymes is limited, but the activation of adenyl cyclase by catecholamines requires at least two molecular loci, one for recognizing and binding the catecholamine and another for catalyzing the formation of cyclic-AMP (5). Research suggests that the liquid-binding component is a distinct molecular entity from the adenyl cyclase catalytic activity. Evidence has demonstrated that receptor function and catalytic activity can be separately disrupted by chemical or enzymatic methods (71,115). Genetic studies have revealed that the adrenergic receptor and adenyl cyclase are products of separate genes (52). In addition, studies on solubilized membrane preparations have demonstrated that the receptor and adenyl cyclase activity partition independently during gel exclusion chromatography (73).

Stimulation of isolated cells with an increase in intracellular cyclic-AMP appears to be temporary, although the proposed effects of cyclic-AMP such as stimulation of lipolysis with release of glycerol and free fatty acids are sustained (80). The reduction of cyclic-AMP after an initial peak may be related to formation of feedback regulator (46, 47), involving the release of adenosine (31,106) or to activation of phosphodiesterase by lipolytic agents (94,132). These lipolytic reductions differ from the cyclic-AMP lowering and antilipolytic actions of insulin (107,132).

A relationship between intracellular adipose cyclic-AMP concentrations and rate of lipolysis has been found in nonruminant species (15,39,80). This substantiates the hypothesis that several protein and catecholamine hormones act via the cyclic-AMP system (16,39).

Protein Kinase in Lipolysis

Intracellular effects of cyclic-AMP have been shown to involve activation of a protein kinase which modifies enzyme activity by phosphorylation (49,62). The various properties of adipose protein kinase have been investigated (24,26,59) and alterations in activity of intact cells have been shown to parallel cyclic-AMP concentrations under influence of epinephrine (113). There is an apparent dilemma relating the properties of isolated protein kinase to perceptible intracellular cyclic-AMP concentrations, which has led researchers to propose that cyclic-AMP may be compartmentalized intracellularly with a fraction sequestered and metabolically inactive (64,93).

Researchers have demonstrated a physiological regulatory role of protein kinase to activate hormone sensitive lipase enzyme (113). Epinephrine addition to rat fat pads caused an increase in cyclic-AMP dependent kinase activity. These activations were associated with the conversion of the inactive holoenzyme to the active catalytic subunit form. The time course for the formation of the catalytic subunit to protein kinase paralleled the epinephrine induced increase in cyclic-AMP concentration.

Hormone Sensitive Lipase

The prompt hydrolysis of adipose tissue triglycerides which result in glycerol and free fatty acid cellular release, is initiated by the activation of hormone sensitive lipase (117,122). Lipase has been investigated in homogenates and partially purified preparations, but the activation by hormonal stimulation is greatest in intact cells. Lipase from rat adipose tissue is stimulated 50 - 100% by addition of ATP. Cyclic-AMP and protein kinase (25,42,59,100) activation is paralleled by phosphorylation of lipase (59) and activation is not possible when lipase is isolated from tissue previously stimulated with epinephrine (42).

Beta-Adrenergic Receptors

Investigations of catecholamines as biological agents have shown responses as diverse as glycogenolysis in liver, aggregation of platelets, contraction of myometrium, increased contractility of heart and lipolysis in adipose tissue. For many years researchers have attempted to elucidate the manner in which catecholamines interact with responsive tissues. Research in the area of lipolysis has formulated the concept that catecholamines initiate biological response by first interacting with discrete sites on the cell membrane.

Early investigations (22) into the action of catecholamines in 1937 have brought about the postulation that the initial interaction takes place at a specific cell structure termed "receptor". The response is proportional to the number of receptors occupied by the catecholamine (127).

The adrenergic concept was extended by an investigator (2), who performed a pharmacologic study of the responses of a variety of tissue to a series of structurally different catecholamines. The results demonstrated a response based on order of potency of the agonists in eliciting the response. It was concluded that there were two types of catecholamine receptors, he termed "alpha" and "beta" based on their excitatory or inhibitory functions on tissues.

In further studies, the receptor concept was expanded by the use of antagonists that could selectively inhibit either alpha or beta adrenergic responses (5). However, the utilization of pharmacological agents in the study of beta-adrenergic receptors was limited and researchers turned to new techniques, such as radioligand binding studies. One of the earliest studies with a radioactive compound to study beta-adrenergic receptors was reported in 1967 (95). Investigations showed that the radioactive antagonist $[^3H]$ propranolol bound to pieces of guinea pig muscle <u>in vitro</u>. However, under these conditions binding appeared to be nonsaturable and not characteristic of a beta-adrenergic receptor.

Workers (127) state that if the binding of radioactive ligand is representative of the binding to physiologically relevant beta-adrenergic receptors, then a number of criteria should be met. These criteria require that the radioligand be pure and biologically active and that the binding sites be saturable, of high affinity, and of the appropriate specificity and stereospecificity. In addition, the kinetics of the binding must be consistent with the kinetics of its biological effect.

During the period between 1967 and 1974 several research teams began investigating the possible use of radiolabeled beta-adrenergic agonists such as [^{3}H]-isoproterenol, [^{3}H]-epinephrine, and [^{3}H]-norepinephrine to identify receptors. The binding of these radiolabeled beta-adrenergic antagonists was studied in rat tissue membranes from adipose tissue (54), liver (82,120) heart (67) and turkey crythrocytes (12). The binding sites did not, however, have the binding characteristics expected of beta-adrenergic receptors as previously stated (127). The binding was nonstereospecific, and competitive beta-adrenergic antagonists inhibited binding only at very high levels. The sites labeled by the catecholamines apparently were not beta-adrenergic receptors and the exact nature of these "nonreceptor" binding sites is unknown.

Researchers stated (127) that previous to 1974 there were no reports of successful identification of beta-adrenergic receptors using radioactive ligands. However, this time frame saw three groups develop methods that ostensibly permitted direct study of beta-adrenergic receptors. The three groups all used potent beta-adrenergic antagonists, [^{3}H] propranolol (6,70), (-) [^{3}H] dihydroalprenolol (68) and [^{125}I] hydroxybenzylpindolol (7). During 1975 and 1976 the techniques were applied to a variety of catecholamine sensitive mammalian systems (3,4, 19,78,85,125,129,131) which also proved useful in studying solubliza-tion of the receptor (21). Site-site interactions among receptors (72) and ligands were examined along with alterations of receptor number caused by a variety of physiological manipulations (40,43,56,66,79,84,

86,87,88,108 ,128). Additionally, beta-adrenergic receptor research has examined alteration of the receptor affinity caused by nucleotides (69), identification of ectopic beta-adrenergic receptors (124), and genetic regulation of receptors in various cell clones (78).

Physiological Regulation of Beta-Adrenergic Receptors

Investigations into ruminant adipocyte beta-adrenergic receptors and their physiological regulation does not appear to exist in the literature. However, a mammalian adipocyte beta-adrenergic receptor reports do exist and allow the assumption that these receptors are also found on dairy cows adipocytes. Beta-adrenergic receptors and their physiological regulation in dairy cows is important for future attempts to increase milk production and control related metabolic diseases (fat cow syndrome, ketosis) that effect fat tissue and lipid mobilization. The following is a review of physiological regulation of beta-adrenergic receptors.

Research indicates that considerable evidence exists that the physiological properties of adrenergic receptors, and hence adrenergic responses, can be regulated by a variety of agents. Frogs injected with beta-adrenergic catecholamines produced a conformational change and refractoriness of the erythrocyte membrane adenyl cyclase to subsequent stimulation in vitro with isopreterenol (84,86,87). The decrease in adenyl cyclase activity was accompanied by a similar 68% fall in the number of beta-adrenergic receptors. There was, however, no change in affinity of the ligand-receptor binding sites on the erythrocyte membranes. Catecholamine antagonist propranolol was used

to block the desensitized effects of isopreterenol and results indicated a return to catecholamine sensitivity and receptor number when propranolol was injected into previously desensitized animals (84). The conformational change in adenyl cyclase beta-adrenergic receptor association could be the result of a primary change in the fat-cell membrane itself. Reports of masking, burying in the membrane or desensitization of adenyl cyclase beta-adrenergic receptor complex, appear to be associated with the conformational change and refractoriness of fat cells to catecholamines (40).

Conformational change of the receptor-adenyl cyclase coupling mechanism was reported (126) to be partially explained by the formation of a high affinity desensitized state of the receptor by a catecholamine agonist. The agonist induced loss of receptors and desensitized state was demonstrated in frog erythrocyte membranes with the agonist $[^3H]$ hydroxybenzylisoproterenol (126). Similar reports of desensitization of frog erythrocyte beta-adrenergic receptors by agonists have also been described (88) with a decrease (-) $[^3H]$ dihydroalprenolol binding to desensitized receptors. The (-) $[^3H]$ dihydroalprenolol binding sites were rapidly resensitized by <u>in vitro</u> incubation with guanosine 5'- (beta, alpha-imino)-triphosphate (88). Research with guanosine nucleotides, indicates a possible conformational change in the beta-adrenergic receptor adenyl cyclase coupling during desensitization by the catecholamine agonist. The guanine nucleotides appear to cause a favorable conformational change at receptor-adenyl cyclase coupling with resensitization of the beta-adrenergic receptor.

Similar alterations in receptor number and adenyl cyclase desensitization have been demonstrated in the rat pineal (56) and lymphoma cells (108). The utilization of isopreterenol (56,108) in mutant cells and in pineal cells as an agonist to induce refractoriness appears to require an intact adenyl cyclase. Adenyl cyclase is said to exert its regulatory controls on beta-adrenergic receptors in addition to generation of cyclic-AMP (127).

High concentrations of catecholamines have been shown to depress the number of beta-adrenergic receptors (56,108). Likewise researchers (79,114,128) have demonstrated an increase in receptor numbers by the removal and lowering of catecholamines in blood and tissue. Reports indicate increased beta-adrenergic receptor number and supersensitivity of receptors to catecholamines with injection of 6-hydroxydopamine in rats (114). Injections of 6-hydroxydopamine caused a decrease in tissue catecholamine levels. One week after 6-hydroxydopamine injections, rat brain tissue, exposed to isopreterenol, had cyclic-AMP accumulations 80% greater than controls. Additions of $[^{125}I]$ hydroxybenzylpindolol to rat brain tissue demonstrated a supersensitivity with a 31% increase in number of beta-adrenergic receptors with no change in apparent binding affinity of ligand to the receptor (114).

Investigators have reported that excess thyroid hormones (hyperthyroidism) will cause an increase number of beta-adrenergic receptors. Exogenous additions of thyroxine on rat myocardium (128) and rat adipocytes (79) caused increased beta-adrenergic receptor numbers and enhanced catecholamine sensitivity. Affinity of binding sites for the

beta-adrenergic agonist isopreterenol was measured with a competitive affinity study with (-) $[^3H]$ dihydroalprenolol (128). The relative binding affinity of isopreterenol in control and hyperthyroid myocardial tissue was nearly identical. Thyroid treated animals had a significant increase in number of beta-adrenergic receptors with no change in receptor binding affinity for agonist or antagonist (128).

Researchers working with adipose tissue from animals with altered thyroid states have reported severely reduced lipolytic sensitivity of fat cells to epinephrine from hypothyroid rats. The lipolytic response of fat cells from hypothyroid rats was restored to control levels by administration of thyroid hormone (79).

Influence of growth, cell size and aging on beta-adrenergic receptors has been examined in rat fat cell membranes. A study of binding (-) $[^3H]$ dihydroalprenolol to crude adipocyte membranes from rats of different ages has reported (20,40) that age and cell size failed to modify binding kinetics, the reversibility and the stereospecificity of (-) $[^3H]$ dihydroalprenolol on fat cell membrane receptors. The dissociation constant $[K_d]$ of these receptor sites was not affected by age and varied from 15 to 20 nM. In contrast, the maximal number of beta-adrenergic receptors varied with age and cell size. The number of receptor sites increased proportionally to fat call surface and with maturation (1-8 months of age) of animal. However, senescence (12-30 months of age) depressed the number of beta-adrenergic receptors although cell size remained constant throughout this period. These changes

were accompanied by parallel variations in the sensitivity of adenyl cyclase to catecholamines.

Similarly experiments (66) with genetically obese mice have demonstrated an impaired response of adenyl cyclase to stimulation by epinephrine. Investigators have suggested a disturbance may occur in the coupline step between the hormone receptor and the catalytic site of the adenyl cyclase in these obese animals (66).

Lipid Mobilization in Ruminants

Adipocyte lipolysis in the dairy cow involves both subcutaneous and internal fat depots. Fat depots are mobilized at parturition and early lactation in the cow to meet the negative energy balances associated with high milk production. Following is a review of lipid mobilization in the dairy cow.

The major fatty acids in adipose tissue of cattle in order are oleic (C 18:1), palmitic (C 16:0) and stearic (C 18:0) (123). In adult ruminants the dietary unsaturated fatty acids, consisting mainly of linoleic (C 18:2) and linolenic (C 18:3), are hydrogenated in the rumen and consequently the major (C 18) fatty acids absorbed from the intestine are stearic (C 18:0) and oleic (C 18:1) (38). Unsaturated fatty acids have been reported to account for 50 to 60% of the total fatty acids (27). The percentage of unsaturated fatty acids is higher in subcutaneous adipose tissue than in the perirenal depot (123) and increase as cattle increase in weight and (degree of finish) fatness (74). Stearic acid (C 18:0) decreases in subcutaneous adipose tissue as animals increase in age, whereas, oleic (C 18:1) increases (27).

Biphasic changes in adipose function associated with parturition have been reported in various species (11,50,61). Early pregnancy is characterized by lipid deposition while lipid mobilization predominates during late pregnancy and at parturition. Dairy cows in late pregnancy and early lactation have demonstrated a mobilization of body fat to meet caloric requirements (14,35).

Increased lipid mobilization in late pregnancy has been demonstrated in the ewe (90). Plasma lipid values in ewes measured three days prior to parturition were markedly increased (34%) over the total lipid observed at the 100th and 180th days after parturition respectively.

Blood lipid patterns and adipose lipogenesis, lipolysis and enzyme pattern changes have been recorded during pregnancy in non-ruminants and are useful in comparison to dairy cows. Investigators report two to four days prior to parturition the triglycoride fatty acids in plasma of rats increased, hormone sensitive lipase decreased, with no change in food consumption or dietary fat. Presumably during late gestation lipolysis is caused by the hormonal changes associated with parturition (60,61,91).

Mobilization of lipids to meet caloric requirements has been examined in one high producing cow with milk levels over 50 kg daily in early lactation and with a yield of approximately 8800 kg milk in a 305 day lactation (35). Measurements of the above cow indicated she was in negative energy balance the first 12 weeks with critical deficiencies the first four weeks drawing on body energy stores. The

average net energy balance was -206 Mcal/day for 62 days of lactation which would require mobilization of over 2 kg/day of body fat. During late lactation this animal was in positive energy balance storing energy as adipose tissue for her next lactation.

Lipid mobilization in early lactation is also accompanied by depletion of triglycerides in adipose cells (28), elevated blood nonesterified fatty acids and decreasing blood nonesterified fatty acids at peak lactation (97). The rise in blood lipid mobilization and a drop in blood lipids with increasing levels of milk production, presumably reflects increased removal by the mammary gland (9,91).

Bovine milk contains approximately 3 - 4% milk fat. The source of these milk fatty acids are either dietary or synthesized de nova. Under in vitro conditions mammary tissue of the rat and cow synthesizes fatty acids by the malonyl-CoA pathway. This method of fat synthesis requires NADPH, which is largely supplied by the pentose phosphate pathway. This in turn has been reported to account for the large quantities of glucose oxidized by mammary glands during lactation (55, 76). The cow however, is not able to incorporate glucose into fatty acids as the rat, but can only utilize carbon from plasma acetate and beta-hydroxybutyrate which arises from fermentation of dietary carbohydrates in the rumen (36). It has been documented that, with these short-chain fatty acid precursors, a high percentage of the dairy cow milk fat is composed of fatty acids containing 4-14 carbon atoms which give butter its characteristic flavor (37).

Measurements of arteriovenous difference of lipid across the udder of cows (41) have been known for over 40 years, but an estimate of milk fatty acids that are transferred from the plasma as contrasted with those synthesized de nova (75) is not accurately known. The udder removes large quantities of acetate, beta-hydroxybutyrate, free fatty acids (FFA), free glycerol, and triglyceride from chylomicron and low-density lipoprotein -- fractions of plasma lipids. Acetate and beta-hydroxy butyrate are known to be synthesized into most milk fatty acids with lengths up to myristate (C 14) and about 50% of the palmitate (C 16). These, however, represent only 40% by weight of total milk fatty acids. The remainder which are long chain acids, mainly sterate and oleate (C 18) have been shown by isotope studies to be taken up from the plasma both from free fatty acids and triglyceride fractions (75).

The stress of early lactation in the dairy cow and her ability to mobilize body fat to meet caloric requirements involves both endocrine and enzymatic systems. Metabolic and enzymatic changes at the onset of lactation has been measured in adipose slices. A change in lipogenic activity was reported by increased acetate incorporation into adipose slices (8). Adipose lipoprotein lipase activity, which has the function of increasing uptake of blood lipid into adipocytes, was detected in early lactation (109). Injections of norepinephrine into lactating cows caused a greater increase in blood glycerol and nonesterified fatty acid levels than in nonlactating cows (112).

Lipogenic enzyme patterns indicate only negligible changes in activities when measured during lactation (8). Information concerning

chronic or acute hormonal changes that produce effects in bovine adipose function are limited. Prolactin has been implicated with a depression in lipoprotein lipase activity during lactation (34).

In vitro incubations of subcutaneous adipose tissue from cattle and sheep with epinephrine and norepinephrine resulted in less than a one to four fold increase in lipolysis (1,58,130). This response is small in magnitude when compared to that observed with rat adipose tissue in the presence of epinephrine. Here the response was a 10 to 60 fold increase (96,102,103). When cattle and sheep have been injected with large doses of epinephrine and norepinephrine, only minor increases in plasma FFA levels were observed (1,58,111). Lipolytic capacity of dairy cow adipose tissue as measured in vitro have indicated increased glycerol and free fatty acid release from adipocytes incubated with and without epinephrine (130).

Glucose is reported to inhibit the release of FFA from adipose tissue in ruminants in vivo, as well as in vitro, probably by stimulating the reesterification of fatty acids (121). Similar results have been obtained with sheep in vivo (57). However, investigators (83) have shown that glucose (3.5 mM) addition did not significantly affect fatty acid or glycerol release from bovine adipose tissue in the presence or absence of norepinephrine. Additionally, it was shown that sodium butyrate (10 mM) and sodium-DL-beta-hydroxybutyrate (10 mM) significantly inhibited lipolysis in bovine subcutaneous adipose tissue in vitro (83). The butyrate effect was observed in both control and norepinephrine stimulated studies.

A ruminant adipose tissue study has been reported utilizing glucagon, estradiol and norepinephrine to elicit in vitro lipolysis. This report indicates that glucagon and estradiol do not cause lipolysis in ruminants. Epinephrine, however, produced a significant increase in FFA and glycerol release from the adipose tissue as compared to controls without epinephrine additions (29).

Bovine dependence on large releases of cellular lipid to meet lactational requirements is well documented (35). The potential sites of hormone action in dairy cow adipose is limited and the function of beta-adrenergic receptors in initiating the cascade release of tissue lipid is largely unknown. Research has demonstrated that the number of receptor sites per cell may vary with diet and physiological state (40, 43,56,66,84,86,87,88,108,128). Thyroid hormone and growth hormone appear to act, in part, by increasing amounts of adenyl cyclase activity in adipose tissue (63,79,101).

Increased adenyl cyclase levels presumably lead to increased adipose sensitivity and responses to hormonal stimuli. Thus far, investigations with ruminants have not been extensive enough to allow definitive conclusions on the endocrine role of beta-adrenergic receptors on bovine adipocytes. Figure 1 provides a summary of proposed lipolytic function in ruminants.

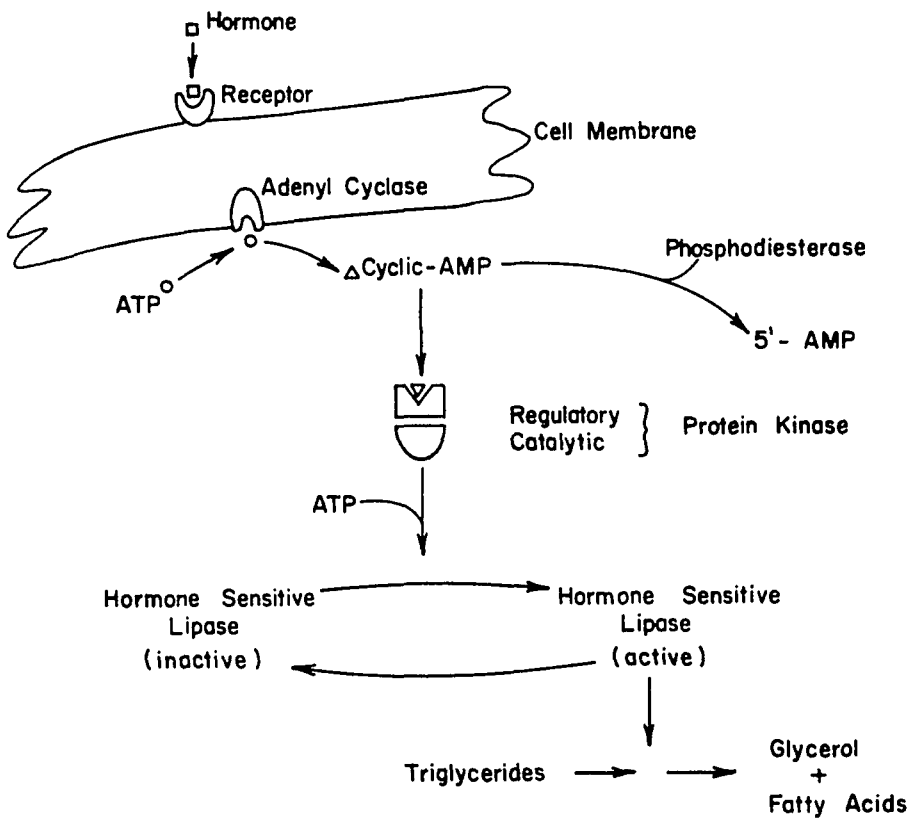

Figure 1. Action of catecholamine and peptide hormones upon adipocyte membrane receptors and lipolytic function

CHAPTER 3

PROCEDURE

Experimental Animals

Eight Holstein cows were selected from the University of Arizona Dairy Research herd and served as the source for adipose tissue. Cows were chosen for milk production (6500 - 9000 kg), age (4 - 6 yr) and stage of gestation (250 days). Body weights were measured 30 days prior to calving (250 days gestation), at parturition, and 30 days postpartum to assess changes associated with early lactation. All cows were biopsied for adipose tissue at 30 days prior to calving and 30 days postpartum. The experiment was conducted from June 1 to August 31, 1979.

Cows were group fed a dry cow ration containing alfalfa hay (1/5 baled, 4/5 cubes) or a lactating ration containing a 50:50 ratio of concentrate to alfalfa hay (2/3 cubes, 1/3 baled) based on production.

Milk weights and samples were taken during two consecutive milkings, 30 days postpartum. Milk samples were composited and percent milk fat was determined by the standard Babcock Method.

Dairy Cow Adipose Tissue Biopsy

Dairy cow tail head region was selected as the site for subcutaneous adipose tissue biopsy based on a series of pretrial tests and previous work utilizing subcutaneous adipose in dairy steers (29). Prior to biopsy, cows were clipped of hair and scrubbed with a

disinfectant on the tail head. A two percent lidocaine hydrochloride solution was injected into the skin as a local anesthetic. Lidocaine solution was injected into skin to prevent contamination of adipose tissue with anesthetic.

A skin incision approximately 8 cm long was made and 4 g of adipose tissue was removed and placed in physiological saline at 38 C. Pretrial fat samples were found to be sensitive to temperature and degraded rapidly when not maintained at 38 C. The skin was sutured and cleansed after the removal of the fat sample (92).

Preparation and Incubation of Dairy Cow Isolated Fat Cells

Dairy cow isolated fat cells were prepared by published methods (102,127,130). Adipose tissue samples were dissected free of vascular and connective tissue. Four grams of tissue were minced and placed in a 25 ml Erlenmeyer flask with 10 ml of albumen buffer (pH 7.4). Albumin buffer was prepared fresh daily and contained 128mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, 10 mM Na_2HPO_4, 3% bovine serum albumin (Fraction V powder; Sigma Chemical Co., St. Louis, MO), 2.5 mg/ml of crude collagenase (clostridium histolyticum; Worthington Chemical Co., Freehold, NJ). Preparations of buffer were made fresh daily to prevent bacterial contamination due to high concentrations of albumin.

The suspension of albumin buffer and adipose tissue was incubated on a metabolic shaker at 60 cycles per minute, 38 C for 50 minutes. Digestion was terminated by filtering the cells through one layer of cheesecloth followed by centrifugation on a clinical centrifuge for 20 to 30 sec at approximately 1000 rpm. Cells were washed

three times with gentle resuspension in warm (38 C) albumin buffer. Washed cell suspensions were transferred to incubation tubes using a 1 cc syringe affixed to a short piece of surgical tubing (12 cm). Surgical tubing was used to prevent the breaking of large and fragile bovine adipocytes. The volume of isolated cells (approximately 40,000 cells/ml) and albumin buffer in each tube was 950 µl.

To begin each lipolytic study, 50 µl of L-epinephrine bitartrate (Sigma Chemical Co., St.Louis, MO) was dispensed into each incubation glass tube (12 X 75 mm). Final volume of hormone, albumin buffer and isolated fat cells was 1 ml. The concentration of L-epinephrine in the incubation media was 10 µM/ml.

Isolated fat cells were incubated during lipolysis studies on a metabolic shaker water bath at 38 C, 30 cycles per minute. Incubations were terminated with 0.1 ml of 2N HCl prior to placing tubes in a boiling water bath for 1 minute. Incubation tubes were allowed to cool and .05 ml of 4N NaOH was added to each. After centrifugation (1000 rpm) of tubes, 100 ul aliquots were removed for determination of total cyclic-AMP. Additional aliquots (15 µl) were used for total glycerol determination. All samples were stored at -20 C until assayed. Bovine lipolytic samples were not affected by freezing and thawing (29).

Glycerol Analysis

Fifty microliters of L-epinephrine bitartrate was added to cell suspensions prepared as previously mentioned. The final concentration of epinephrine was 10 µM/ml. Following incubation, glycerol released

into the media was assayed by Glycerol Reagent Kit (Calbiochem Corp., La Jolla, CA). Glycerol was quantitated by the following oxidation-reduction reaction.

1. Glycerol + ATP $\xrightarrow{\text{Glycerol Kinase}}$ - Glycerol Phosphate + ADP
2. ADP + Phosphoenol pyruvate $\xrightarrow{\text{Pyruvate Kinase}}$ pyruvate + ATP
3. Pyruvate + NADH $\xrightarrow{\text{Lactate Dehydrogenese}}$ Lactate + NAD

The decrease in concentration of NADH was measured spectrophotometrically (Gilford Instrument Lab. Inc., Oberlin, OH) at 340 nm.

Glycerol enzymatic incubations were conducted in 250 μl microliter plates (Dynatech Laboratories, Inc., Alexandria, VA) at 30 C. Standard glycerol curves were prepared using incubation media treated in the same manner as the medium plus fat cell glycerol. Data are expressed in $\mu M/10^6$ cells/time of incubation.

Cyclic-AMP Analysis

Isolated fat cell suspensions were incubated with 50 μl L-epinephrine bitartrate as previously mentioned. The final concentration of epinephrine was 10 μM/ml. Cyclic-AMP formed during incubations was assayed with the New England Nuclear Radioimmunoassy Kit (New England Nuclear, Boston, MA). The labeled antigen was a succinyl tyrosine-$[^{125}I]$ methyl ester of cyclic-AMP in a double antibody system (116).

Standard cyclic-AMP curves were prepared using incubation medium treated in the same manner as the medium plus fat cells taken for analysis (32,33). Radioactive gamma emissions were measured on a Beckman Radioimmuno-Analyzer (Beckman Instruments, Inc., Anaheim, CA). Data are expressed in $pM/10^6$ cells/time (min) of incubation.

Preparation of Dairy Cow Isolated Fat Cells (IFC) for Receptor Binding

Isolated intact bovine fat cells were prepared essentially as described by research reports (23,53). Preliminary experiments were conducted with rat epididymal adipose tissue to establish procedures and techniques for receptor binding. Isolated intact fat cells were washed twice with the Krebs-Ringer Phosphate buffer containing 3% albumin (pH 7.4) and once with 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$ (pH 7.4) at 38 C. The fat cells were resuspended in the Tris buffer (1 ml of buffer plus isolated fat cells) and used directly in the binding assay. Catecholamine antagonist (-) $\left[^3H\right]$ dihydroalprenolol was used in this study because of its high affinity, kinetics, specificity, and stereospecificity for the adipose beta-adrenergic receptor (40,125).

Receptor Binding Assays

Isolated bovine adipocytes (approximately 40,000 to 50,000 cells/100 μl) were incubated with ($1 - 2 \times 10^{-10}$ M) of (-) $\left[^3H\right]$ dihydroalprenolol in total volume of 150 μl of incubation buffer (50 mM tris - HCl, 10 mM $MgCl_2$, pH 7.4) for 12 minutes with shaking at 38 C. In the competition experiments, unlabeled (-) epinephrine was added in buffered solutions to give the indicated concentration in the assays. At the end of incubation 2 ml of ice cold incubation buffer was added to each incubation tube.

Contents of the tubes were rapidly filtered under vacuum through a single nuclepore polycarbonate filter (0.4 μM pore size, 25 mm diameter; Nuclepore Corp., Pleasanton, CA). Filters were then

washed with two 5 ml - aliquots of ice cold incubation buffer to minimize the nonspecific binding. The filters were placed directly into plastic scintillation vials and 10 ml of a 40% Xylene - 60% Quantifluor mixture was added and counted for 50 minutes on a Beckman Liquid Scintillation Counter (Beckman Instruments, Inc., Anaheim, CA). Assay vials were counted in duplicate.

Nonspecific binding to cell protein was determined by measuring the amount of radioactivity retained on filters when incubations were performed in the presence of a high concentration, 10 μM, of (-) epinephrine. Nonspecific binding including counter background, nonspecific absorption to filters, and a very small nonspecific absorption to protein was, in general, 10 to 20% of the total counts bound. The small amounts of (-) $\left[^{3}H\right]$ dihydroalprenolol nonspecifically absorbed to the filters was less than 0.5% of the total counts filtered bound to the filters and was unaffected by the presence of high concentrations (10 μM) of unlabeled (-) epinephrine. Specific binding was determined by subtracting the nonspecific binding from the total counts bound. The results are expressed in $M/10^{6}$ cells.

Fat cells were counted from aliquots taken from the cell suspension diluted with buffer containing albumin. A 5 ml aliquot of dilute suspension containing 10 to 20 fat cells was placed on a hemacytometer (AO Instrument Co., Buffalo, NY) and counted five or more times by visual inspection with light microscope. Cell sizes were also measured at this time with a light microscope fitted with an occular micrometer.

Isolated fat cell weight was estimated by the vacuum filtration of cells (approximately 40,000 cells/ml) through a Whatman glass fiber filter and allowed to dry. Adipocyte weights were determined by analytical balance.

Statistical Analysis

Statistical package for the social sciences (SPSS) was used for the analysis of data (89). Student's t test was used to compare treatment effects. Regression equations were calculated for kinetic binding and dissociation of ligand from its receptor. Pearson correlation coefficients were computed and utilized to compare the relationships of kinetic binding and dissociation of ligand with production variables and lipolytic cell response.

CHAPTER 4

RESULTS AND DISCUSSION

All cows on experiment were observed for distocia at parturition, and metabolic disorders, such as ketosis, milk fever, and fat cow syndrome. None of the animals appeared to experience disease or calving difficulty during the course of the experiment.

The diametric periods of lactational state (milking vs. dry) and its affect on lipolysis and receptor binding are reported in the following results and discussion.

Influence of Dairy Cow Lactational State on the Kinetic Characteristics of (-) [^{3}H] Dihydroalprenolol Binding

Specific binding of (-) [^{3}H] dihydroalprenolol to adipocytes of the lactating cow is rapid (Figure 2), reaching 75% of the equilibrium within the first minute of incubation. Kinetic binding information concerning cows is not available in the literature, however, similar values were obtained with rat adipocytes at one minute of incubation (40,125). Kinetic data from Figure 2 can be used to calculate the rate constant, K_1, for the reaction $R+L \rightarrow RL$ where R represents free receptor, L represents free (-) [^{3}H]dihydroalprenolol, and RL represents the receptor ligand complex. The reaction in Figure 2 is considered to be a pseudo first order reaction since $R/L << 1$ (23). The ligand (-) [^{3}H] dihydroalprenolol was added at a concentration of 5 nM for kinetic

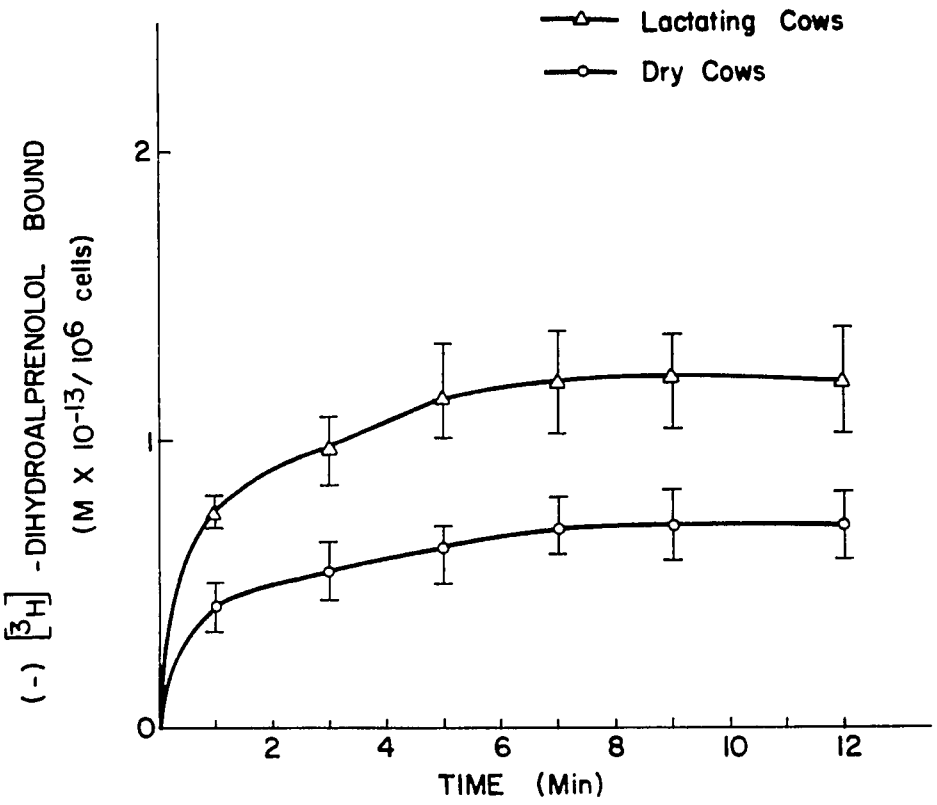

Figure 2. Kinetics of (-) $[^3H]$ dihydroalprenolol binding ($\bar{X} \pm$ s.e.m.) to dairy cow adipocytes

binding studies. Receptor binding values were in range of .05 to .2 pM/ 10^6 cells. Mean values from kinetic binding (Figure 2) were significantly different ($p < .05$) between lactating and dry cows.

Slopes of the lines in Figure 3 (Kob = 0.17 min^{-1} dry vs. 0.21 min^{-1} lactating) provide an estimate of the forward rate constant for the pseudo first order reversible reactions. The second-order affinity rate constant, K_1, for dry cows can be computed from $K_1 = (Kob - K_2)/L = 8.2 \times 10^7$ min^{-1} M^{-1}, where K_2 for dry cows is the independently determined rate constant for the reverse reaction RL $\rightarrow$ R+L (Figure 4). For lactating cows K_1 was 7.2×10^7 min^{-1} M^{-1}. Kinetic rate constants for rat beta-adrenergic receptor studies with adipose tissue reveal similar values ($K_1 = 2.4 \times 10^7$ min^{-1} M^{-1})(125). Results from linear transformation of Figure 2 demonstrate means were not significantly different ($p > .05$) between dry and lactating cows when comparing rate of association of hormone to the beta-adrenergic receptor.

The rate of dissociati n of (-) $[^3H]$ dihydroalprenolol from its receptor (RL $\rightarrow$ R+L) was measured by adding excess unlabeled adrenergic ligand (10 μM epinephrine) to an equilibrated mixture of (-) $[^3H]$ dihydroalprenolol and receptor containing adipocytes (Figure 4). Dissociation at 38 C was rapid following first order kinetics with a rate constant K_2 of 5.8×10^{-1} min^{-1} for dry cows and 5.7×10^{-1} min^{-1} for lactating cows. After the 12 minute incubation with excess unlabeled epinephrine (10 μM) approximately 90% of the specifically bound (-) $[^3H]$ dihydroalprenolol was dissociated in both treatments at 38 C. Results from dissociation means of (-) $[^3H]$ dihydroalprenolol from its

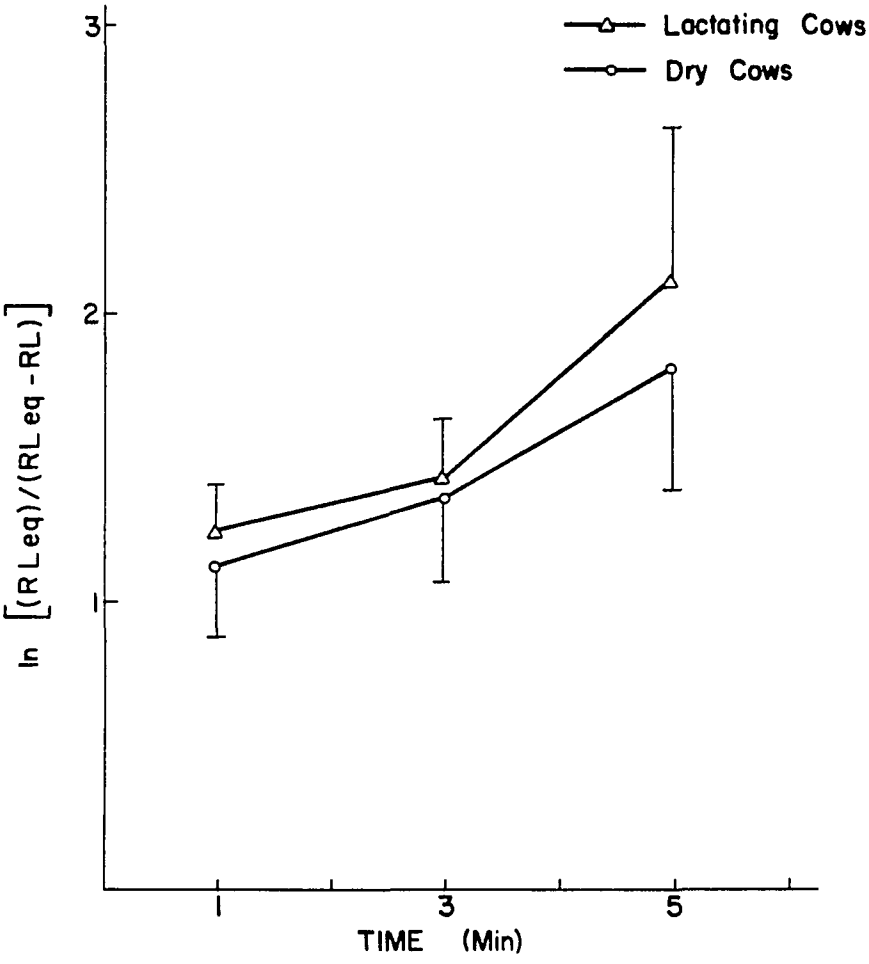

Figure 3. Pseudo first order rate plot ($\bar{X} \pm$ s.e.m./2) of (-) [^{3}H] dihydroalprenolol binding

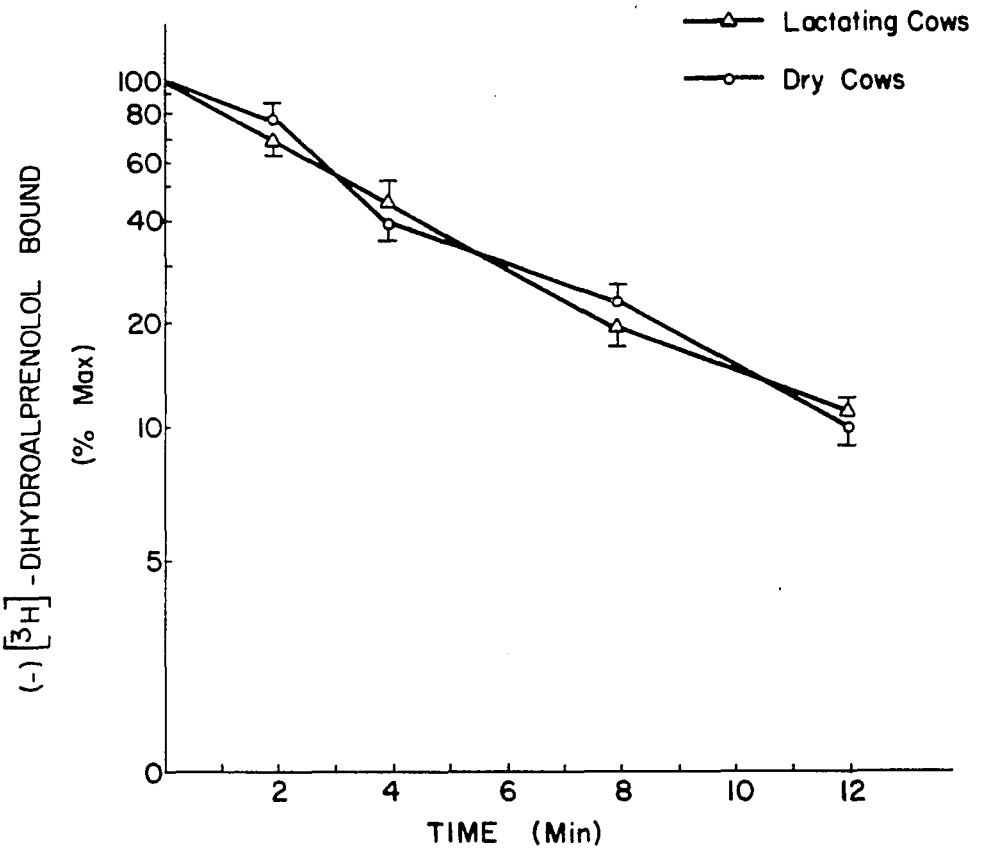

Figure 4. Dissociation of (-) $[^3H]$ dihydroalprenolol ($\bar{X} \pm$ s.e.m./2) from dairy cow adipocytes with 10 μM epinephrine

receptor were not significantly different ($p > .05$) between dry and lactating cows.

Pearson correlation coefficients utilizing dry cow binding kinetics at 12 min (Figure 2) with dissociation kinetics at 12 min (Figure 4) were compared and found significant at ($p < .01$). The results indicate positive relationships between binding and dissociation of (-) $\left[^3H\right]$ dihydroalprenolol from its receptor (r value of .82). These findings indicate the greater the degree of binding, the less dihydroalprenolol dissociated after 12 min of incubation.

Using the rate constant K_2 from Figure 4 and the constant K_1 from Figure 2, the ratio K_2/K_1 equals 7.1 nM for dry cows and was computed as an estimate of the equilibrium dissociation constant, K_d, for the interaction of (-) $\left[^3H\right]$ dihydroalprenolol with its adipocyte binding site. The K_d for lactating cows equals 7.9 nM. These values are lower than the apparent K_d values derived from (-) $\left[^3H\right]$ dihydroalprenolol binding studies with rat adipocytes. Reports of 10 - 15 nM apparent K_d values were demonstrated in rat adipocytes (40,125). Similar values (2 to 10 nM) have been reported for the K_d of (-) $\left[^3H\right]$ dihydroalprenolol in frog erythrocytes (21,85). Comparison of K_d values for dry versus lactating cows of different physiological states demonstrated no difference in binding affinity of cell membrane receptors for the (-) $\left[^3H\right]$ dihydroalprenolol.

Ability of adipocytes to respond to different hormones (thyroxine, insulin and epinephrine) have been shown to be altered by physiological state. Reports suggest that some cellular response

changes may be due to alterations in hormone binding to the cell receptors (40,43,56,66,84,86,87,88,108,128).

The properties of adrenergic receptors and their responsiveness to catecholamines can be regulated by a variety of methods. Investigators (84,86,87) have reported an impaired response to epinephrine by the adenyl cyclase system in frog erythrocytes and adipocyte plasma membranes from old (30 months) rats (40) and obese mice (66). The altered response in obese mice (66) may occur in the coupling step between hormone receptor and the catalytic site of the adenyl cyclase. In these studies, no attempt was made to specifically investigate the influence of old age or the "fat cow syndrome" in dairy cattle on alteration of the adipocyte beta-adrenergic receptor. Fat cow syndromes and other metabolic disorders associated with early lactation are fertile areas for future beta-adrenergic receptor research with dairy cows.

Increases in the apparent number of beta-adrenergic receptors occurred when the dairy cow changes from her dry period to a lactating state (Figure 2). Research with rats suggest a possible internal cellular mechanism for increased receptor numbers at the cell membrane. Researchers have reported that during senescence in the rat there is a marked loss in the apparent maximal number of beta-adrenergic receptors. This loss was not related to cell size which remained constant over this period (40). It is questionable whether a dry cow can be compared to an old rat. However, cows during their early dry periods have minimal production demands and are usually under limited metabolic stress. Initiation of lactation, however, places a sudden heavy demand on the

high producing dairy cow for additional energy intake. If this metabolic energy is not or cannot be met nutritionally, the cow would require mobilization of body fat stores to meet the stress of negative energy intake. Therefore, an increase in beta-adrenergic receptor number when cows enter lactation for increased release of free fatty acids and glycerol is a possibility, because cell response is proportional to the number of receptors occupied by the catecholamine (127).

Influence of Lactational State on Specific Binding of (-)[^{3}H] Dihydroalprenolol to Adipocytes from Dairy Cows

Isolated adipocytes of dairy cows during two different stages of lactation (dry and milking) were incubated for 12 minutes with increasing concentrations of (-)[^{3}H] dihydroalprenolol (2-8 nM). The results are in Table 1. Binding capacity of high affinity dihydroalprenolol binding sites was significantly greater ($p < .05$) in lactating cows than dry cows. However, production period, dry vs. lactating did not significantly ($p > .05$) affect the apparent affinity of these binding sites. Dissociation constants (K_d) derived from kinetic binding experiments were 7.1 nM for dry cows and 7.9 nM for lactating cows, with 5 nM (-)[^{3}H] dihydroalprenolol.

In rats, investigators (125) have demonstrated that adiposyte dihydroalprenolol binding sites are localized in the plasma membrane. Since surface area is a second power function of cell radius we decided to look at cell diameters and fat cell surface variations (Table 2) induced by stage of lactation. The amounts of dihydroalprenolol binding in Table 2 allows comparison of binding from mols per 10^6 cells to mols per mg of cell membrane protein.

Table 1. Specific binding of (-) [^{3}H] dihydroalprenolol ($\bar{X} \pm$ s.e.m.) to dairy cow adipocytes of different lactational states

Conc. of (-) [^{3}H] dihydroalprenolol in assay/10^6 cells	(-) [^{3}H] dihydroalprenolol bound ($MX10^{-13}$)	
nMol	Dry	Lactating
7	.90 ± .14	1.7 ± .25*
6	.79 ± .12	1.5 ± .22*
5	.70 ± .10	1.2 ± .18*
4	.51 ± .07	1.1 ± .15*
2	.35 ± .03	.60 ± .08*

* $p < .05$

Table 2. Influence of lactational state and cell size on dairy cow beta-adrenergic binding sites

	Mean Fat Cell Diameter μm	10^3 x Surface mm^2	Sites/cell	Sites/mm^2 x 10^3
Dry	146	66.9	42,154	630
Lactating	142	63.3	72,264	1141
Rat[a]	77	18.6	31,314	1683

nM of (-) [^{3}H] dihydroalprenolol added to assay	fM/mg cell membrane protein		
	Dry	Lactating	Rat
7	90	170	
6	79	150	
5	70	120	52 = (187)[b]
4	51	110	
2	35	60	

(a) Reference (40)

(b) Increase of the rat cell membrane protein by 360% to equal concentration of cell membrane protein in dairy cow

Mean fat cell size for dry cows was 146 μm versus 142 μm for lactating cows. Statistical analysis indicated no differences in cell size (Table 2) from 30 days prior to calving to 30 days postpartum. Fat cell size did not influence the apparent affinity of beta-adrenergic receptors for (-) $[^3H]$ dihydroalprenolol as indicated in Figure 3.

Investigators (40,79) have provided an estimate of one mg cell membrane protein per 10^6 cells in rat adipocytes. Additions of 5 nM (-) $[^3H]$ dihydroalprenolol were reported to specifically bind 52 fM/mg of protein from rat fat cell membranes (79). Similarly other investigators bound 5 nM of (-) $[^3H]$ dihydroalprenolol to rat adipocyte plasma membrane and provided an estimate of receptor binding of 50 to 70 fM/mg of cell protein (40). Binding of 5 nM (-) $[^3H]$ dihydroalprenolol with dairy cow adipocytes in this study provides an estimate of binding at 70 fM/mg cell protein for dry cows versus 120 fM/mg for lactating cows (Table 2). These values are actually lower than previous (-) $[^3H]$ dihydroalprenolol binding studies with rat adipocytes, when fat cell surface areas are compared (Table 2). Dairy cow adipocyte surface areas are 3.6 times larger than rat. Therefore, 3.6 times as much protein is found in dairy adipocyte membranes. Equating the rat and cow reduces dairy cow beta-adrenergic receptor binding concentrations (fM/mg) in cell membrane protein by the factor 3.6 (Table 2).

Comparison of binding sites per $mm^2 \times 10^3$ (Table 2) reveals greater binding per unit area in rat adipocytes, than dairy cows.

However, the sites per cell were greater in the lactating cow (72) with 5 nM (-) [^{3}H] dihydroalprenolol.

Estimation of cell size and protein distribution in adipocytes of different species is subject to quantitative error. Cell sizes of rats ranged from 40 to 105 μm in diameter (40), while dairy cow fat cell sizes were 100 to 200 μm (48). In addition, the sensitivity of adrenergic receptors on plasma membranes and adenyl cyclase activity may be different between rat and cow adipocytes. Different receptor sensitivity may exist in the transfer of catecholamine receptor binding signal for adenyl cyclase activation and eventually lipolysis in adipose tissue. Incubation of subcutaneous adipose from cattle and sheep with added epinephrine demonstrate a one to four fold increase in lipolysis (1,58,130). This response is small compared to the 10 to 60 fold epinephrine increase with rat adipose tissue (96,102,103).

Experiments have demonstrated occurrance of a wide age-related and size-related increase in the apparent maximal number of beta-adrenergic receptors during the maturation of rats (40). Fat cells increased in size during maturation and researchers reported that as long as fat cells increase in size, composition of the new plasma membrane that is formed remains constant in terms of its content of beta-adrenergic receptors. However, researchers have reported a marked loss in the apparent maximal number of beta-adrenergic receptors during senescence, which could not be related to cell size which remained constant over this period (23).

The ontogenic evolution that appears in rat adipose beta-adrenergic receptors does not appear to be common to all tissues. Studies have reported that the number of beta-adrenergic receptors in rat erythrocyte ghosts progressively declines after 1.5 months of age (20). Conversely, rat cerebral cortex increases until the 20th day of life, after which it progressively declines (44).

The age related and size related changes in dairy cow beta-adrenergic receptors are not known, but reports (48) indicate that bovine subcutaneous adipose tissue may contain smaller cells, both in diameter and volume, than bovine perirenal adipose tissue. This may account for the greater in vitro lipolysis of perirenal adipose tissue in Holstein steers (29). The site difference in lipolysis by Holstein steers could merely be a reflection of number of cells per unit area (29). Larger adipose cells contain fewer cells per unit weight and therefore fewer receptors available for hormone binding on a tissue weight basis. Therefore, all activities associated with adipose lipolysis in this paper are on a per cell basis.

The cause of the post calving increase in dairy cow adipocyte beta-adrenergic receptors (Table 2) may or may not be related to a change in metabolic activity. Lactation is associated with increased energy demands on adipocytes for metabolites which function to maintain high milk production. Mean milk production for the eight cows was 30.6 $\pm$ 2.6 kg/day with 3.08 $\pm$ .57% milk fat at 30 days lactation. Previous research indicates 40 kg/day milk production during early lactation provides an estimate of net energy balance of - 206 mcal/day (35).

It is plausible that adipocytes may increase or decrease in beta-adrenergic receptor bindings depending on milk production. Dry cow loss in beta-adrenergic receptor binding compared with lactating cows could be simply a loss in membrane protein. However, reports utilizing rats indicated no change in protein content of cells with varying receptor binding (40).

In addition to possibility of degradation of the membranes of the beta-adrenergic receptors, this decline during dry period may be the result of adrenergic receptor masking, burying in the membrane or desensitization of receptor sites. Experiments utilizing high concentrations of catecholamines agonists (i.e., isopreterenol, hydroxybenzyl-isopreterenol) during preincubations of beta-adrenergic receptor containing membranes have demonstrated a refractoriness of adenyl cyclase with decreased beta-adrenergic receptor numbers (84,86,87). There may be conformational changes in the beta-adrenergic receptor and adenyl cyclase coupling during desensitization. Desensitization was reversed with guanosine nucleotides and appeared to cause favorable conformational change at receptor-adenyl cyclase coupling (88). On the other hand, the removal and lowering of catecholamines in blood and tissue appeared to cause a super sensitivity of beta-adrenergic receptors and increased adenyl cyclase activity (80). Additions of 6-hydroxydopamine, a known suppressor of tissue catecholamines, caused a 31% increase in number of beta-adrenergic receptors and an 80% increase in cyclic-AMP with isopreterenolol additions to rat brain tissue (80). In all experiments with altered receptor sensitivity, no reports of alterations in kinetic binding affinity have been reported.

It is possible that the altered sensitivity of receptor binding with (-) $[^3H]$ dihydroalprenolol to lactating dairy cow adipocytes in comparison with dry cow binding is secondary to lactation. Induced alterations by one or more hormonal factors (e.g., thyroid hormone, growth hormone, corticosteroids) are known to modify the responsiveness of the fat cells to catecholamines. In addition, excess thyroid hormones will cause an increased number of beta-adrenergic receptors in rat adipocytes (79). Increased receptor numbers and catecholamine sensitivity were also reported with exogenous additions of thyroxine on rat myocardium with no alteration in kinetic binding affinity of (-) $[^3H]$ dihydroalprenolol for adrenergic receptors (128). Conversely, reports of hypothyroid rats exhibiting decreased lipolytic sensitivity to exogenous catecholamines have been cited (79). Hypothyroid lipolytic conditions in rats were returned to control levels by administration of thyroid hormone (79).

The role of thyroid hormone in maintenance of lactation in dairy cattle is well documented (13). Administration of thyroxine to lactating cows usually results in increased milk production and a loss in body weight (fat) of considerable magnitude unless extra feed is provided (13). The effect of altered beta-adrenergic receptor numbers and thyroid status was not studied in this research, but is a distinct possibility and certainly the subject for future research. In the current study, body weights were recorded at three periods, 30 days prior to calving (766 $\pm$ 63 kg), after parturition prior to turning into the milking herd (677 $\pm$ 48 kg) and 30 days post calving (634 $\pm$ 57 kg).

These values indicated that cows as a group lost weight at the rate of 1.1 kg/day during the first 30 days postpartum. In previous studies maximum milk production and greatest net energy balances occurred in a cow 60 to 90 days post calving (35). The cows in this research study may not have reached maximum milk production levels or net energy deficients at 30 days into lactation.

Dairy cows have been documented to mobilize lipids in early lactation to meet demands of high milk production (35). Measurements of cows' weight loss during caloric insufficiency provided an estimate of net energy balance of -206 meal/day for 62 days of lactation. This would require the mobilization of 2 kg/day of body fat by lipolytic hormones. It was also reported that during late lactation this animal experienced a positive energy balance to replace fat stores depleted by energy demands of early lactation (35).

It is possible, therefore, that dairy cows with high circulating levels of lipolytic and lactogenic hormones during early lactation may cause an alteration of beta-adrenergic receptor with increased catecholamine supersensitivity. Alterations in beta-adrenergic receptors may be simply an unmasking or conformational change of the receptor with no change in total membrane protein. The answer to the previous question awaits further research.

Pearson correlation coefficients utilizing dry cow body weights with binding and dissociation kinetics at 12 minutes (Figures 2,4) were significant at $p < .01$. These results indicate positive relationships between dry cow body weights, binding kinetics and dissociation

kinetics. The statistical r values were .83 and .80 respectively. It appears, from these correlations, that the heavier dry cow bound more (-) $[^3H]$ dihydroalprenolol to the beta-adrenergic receptor. In addition, the concentration of (-) $[^3H]$ dihydroalprenolol that remains after in vitro dissociation was greatest in the heavier dry cows.

Influence of Lactational State on Cyclic-AMP Responsiveness to Epinephrine

The addition of 10 μM epinephrine to isolated dairy cow adipocytes increased cyclic-AMP activity very rapidly and then gradually returned to control values (Figure 5). Highest concentrations of cyclic-AMP were observed after 4-5 minutes of incubation. At this time cyclic-AMP concentrations were all significantly higher ($p < .05$) with added epinephrine (10 μM) than in corresponding incubations without epinephrine. Mean cyclic-AMP concentrations with epinephrine were measured at 5, 10, and 120 minutes in both lactating and dry cows. There was no significant difference ($p > .05$) recorded between treatment groups at these times. Additionally, maximal stimulated cyclic-AMP levels at 5 and 10 minutes were subtracted from their controls respectively. These subtracted values are designated epinephrine induced cyclic-AMP concentrations. There was no significance in 5 or 10 minute epinephrine induced cyclic-AMP concentrations between dry or lactating cows.

Cyclic-AMP maximal response to 10 μM epinephrine additions in adipocytes was approximately 250 pM/10^6 cells. The concentration of 10^6 cells corresponded to approximately 1 gm of dairy cow adipocytes.

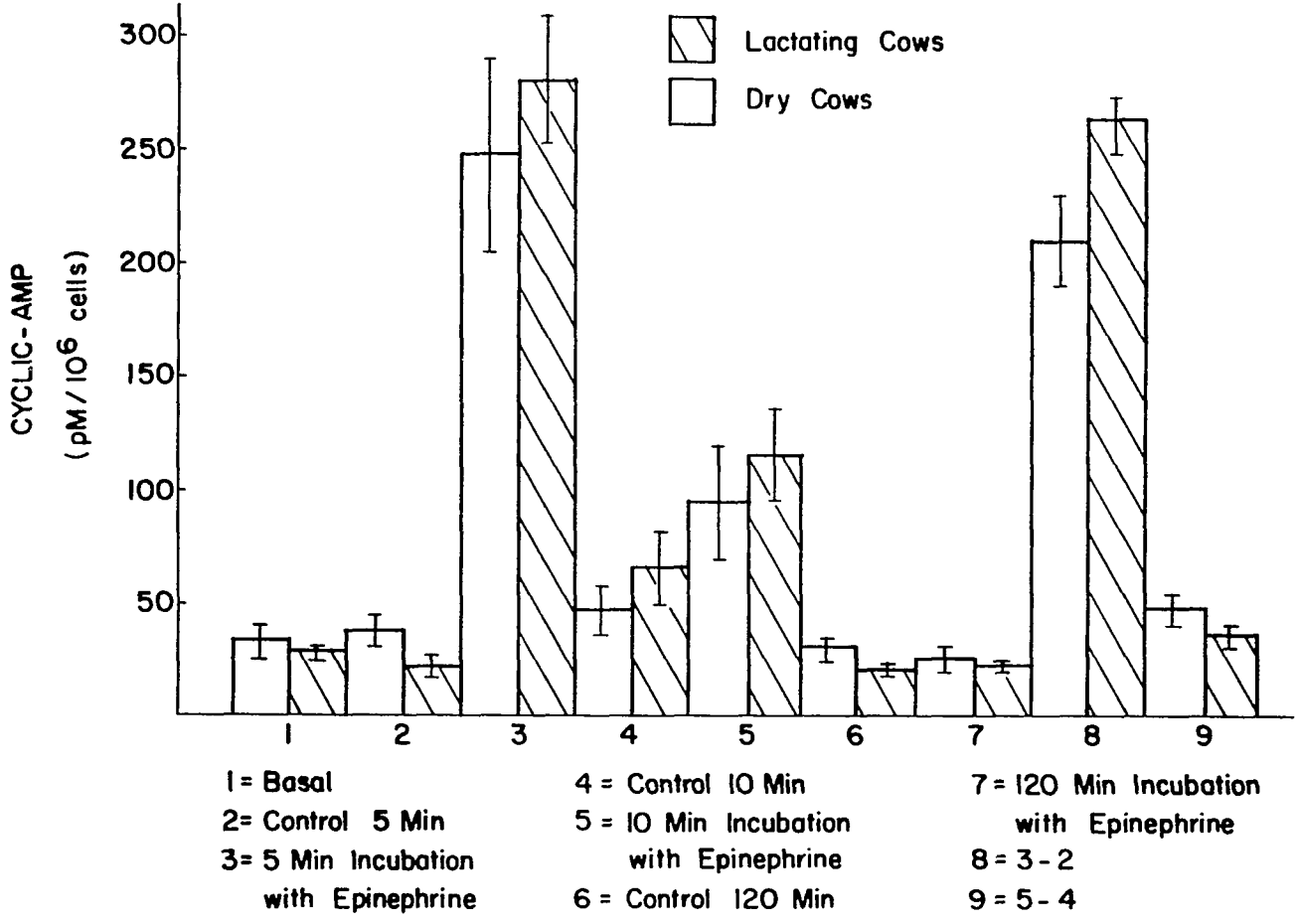

Figure 5. Cyclic-AMP concentrations ($\bar{X} \pm$ s.e.m.) in dairy cow adipocytes during incubation with and without 10 μM epinephrine

Previous reports utilizing rat adipocytes with additions of 10 uM epinephrine to incubations indicate higher cyclic-AMP response and lipolysis. Epinephrine stimulated cyclic-AMP production at 5 minutes in rat adipocyte incubations varied from 400 to 1200 pM/g (17,30,101). These values are large in comparison to the epinephrine effect on dairy cattle adipocyte lipolysis reported in this study.

One of the objectives of this study was to verify the presence of lipolytic mechanisms in the ruminant involving cyclic-AMP. Epinephrine has been shown to increase the activity of adenyl cyclase in the non-ruminant (101). The lipolytic increase in cyclic-AMP and glycerol in this study (Figures 5 and 6) was the result of epinephrine binding to a beta-adrenergic receptor and adenyl cyclase activation with cyclic-AMP being synthesized. Thus, the addition of 10 μM epinephrine to dairy cow adipocytes demonstrates the involvement of cyclic-AMP as the mediator of lipolytic response in the ruminant.

Influence of Lactational State on Glycerol Release in Response to Epinephrine

The addition of 10 μM epinephrine to isolated dairy cow adipocytes stimulated cellular release of free glycerol. Basal and stimulated *in vitro* rates of lipolysis for dairy cow adipocytes of differing lactational states are shown in Figure 6. Data from Figure 6 show that maximal stimulated glycerol production in the dry cow ($2.01 \pm .25$ μM/10^6 cells/120 min) was significantly lower ($p < .01$) than maximal stimulated glycerol production in the lactating cow ($3.91 \pm .23$ μM/10^6 cells/120 min.). There was also significant difference ($p < .05$) between dry and lactating groups (Figure 6) when mean control 120 minute

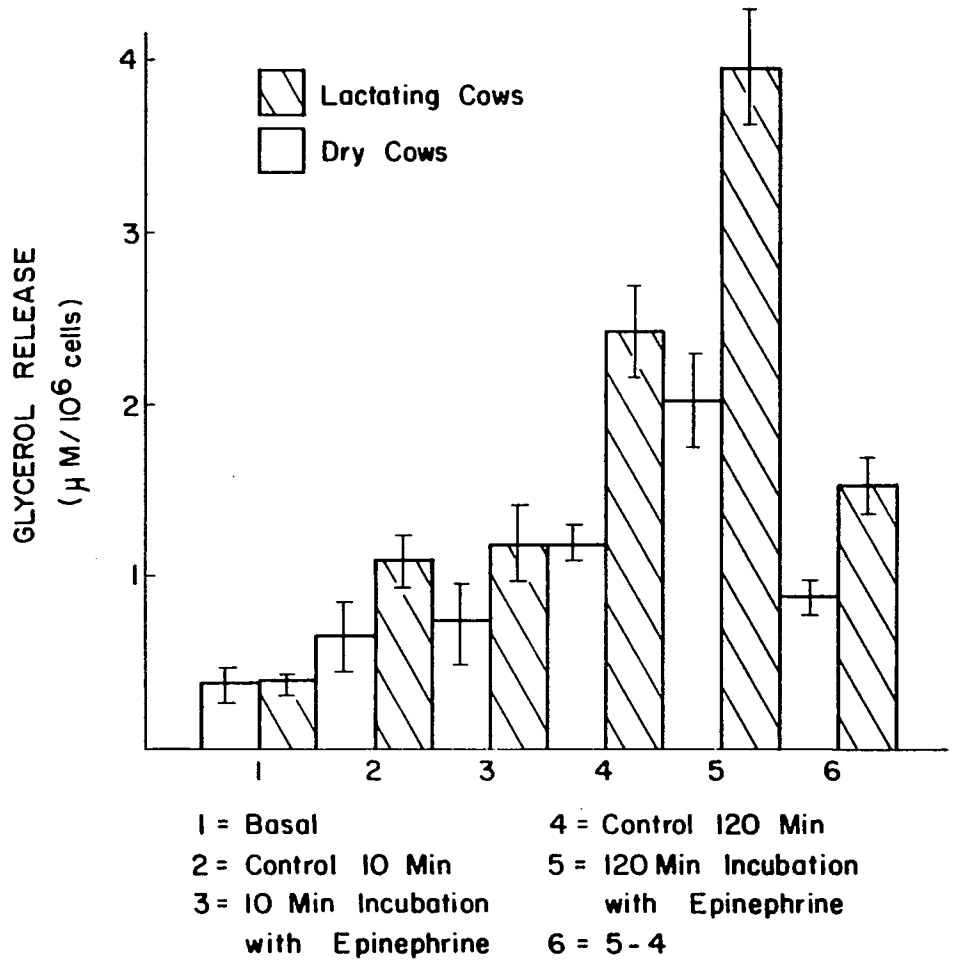

Figure 6. Glycerol release ($\bar{X} \pm$ s.e.m.) from dairy cow adipocytes incubated with and without 10 μM epinephrine

values are subtracted from stimulated values. These subtracted values are designated term epinephrine induced glycerol concentrations. In addition, there was no significant difference ($p > .05$) in dry and lactating cows glycerol production at 10 minutes of maximal stimulation with epinephrine.

Pearson correlation coefficients utilizing lactating dairy cow binding kinetics at 12 minutes (Figure 2) with stimulated in vitro adipocyte glycerol production at 120 minutes (Figure 6) was significant at $p < .05$. The results indicate positive relationships between a lactating cow's ability to bind maximal (-) $[^3H]$ dihydroalprenolol to her beta-adrenergic receptor and maximal stimulated adipocyte glycerol release ($r = .79$). Also, correlation coefficients utilizing milk production with in vitro stimulated adipocyte glycerol release at 120 minutes was significant at $p < .05$. These results indicate positive relationships between milk production and glycerol release from stimulated adipocytes ($r = .70$). As milk production levels increase there was also increased in vitro release of glycerol from adipocytes stimulated with 10 μM epinephrine.

Comparing the results from epinephrine additions to dairy cows and to rat adipose tissue resulted in a 10 to 60 fold increase in rat adipose lipolysis (96,102,103). These values for rats are larger than levels recorded in this study for dairy cows. Maximal stimulated glycerol release in lactating cows at 120 minutes reached 4 $\mu M/10^6$ cells and corresponded to an approximate one fold increase in lipolysis (Figure 6). Glycerol values reported in this study compare

favorably with other subcutaneous adipose tissue incubations in ruminants. Adipocyte research has shown that epinephrine results in a one to four fold increase in ruminant lipolysis (1,29,58,130).

Summary

Recent development of methods using (-) $\left[^3H\right]$ dihydroalprenolol as a ligand for the identification of beta-adrenergic receptors in rat fat cells (48,120) have led this researcher into investigations of varying lactational states and catecholamine receptor function in dairy cattle adipocytes.

Specific binding at equilibrium of (-) $\left[^3H\right]$ dihydroalprenolol to dairy cow adipocytes was greater in lactating than dry cows (.12 vs. .075 pM/10^6 cells) during kinetic studies. On the other hand, no differences were observed in the rate of association or dissociation of the (-) $\left[^3H\right]$ dihydroalprenolol from the adipocyte beta-adrenergic receptor or dry or lactating cows. Estimates of the equilibrium dissociation constant, K_d, for the adipocyte receptor were 7.9 nM in lactating and 7.1 nM in dry cows.

Dairy cow adipocytes were approximately 145 μM in diameter, with mean surface areas of 65 mm^2 X 10^3. Receptor sites per cell were 42,154 to 72,264 for dry and lactating cows respectively. Cows during early lactation lost weight, (1.1 kg/day) and averaged 30.6 kg/day milk production.

Cyclic-AMP values did not vary between lactational states, however, glycerol release from the adipocytes treated with epinephrine was greater in lactating than dry cows (3.91 vs. 2.0 μM/10^6 cells).

The dramatic difference in dry and lactating cow (-) [^{3}H] dihydroalprenolol binding and stimulated glycerol release reflects changes at the beta-adrenergic receptor. Hormone-receptor binding and the transduction of this signal to internal lipolytic enzymes appears greater in the lactating versus dry cow. Increased beta-adrenergic receptors per cell explain the mobilization of greater amounts of glycerol and FFA to meet negative caloric intakes associated with early lactation. Certainly future investigations of this phenomenon as related to factors that alter beta-adrenergic number would greatly aid an understanding of lipolysis associated with early lactation and related metabolic disorders.

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