

# **Metabolic Studies in Cattle using the Hyperinsulinemic Euglycemic Clamp Technique**

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*Ne Cede Malis Sed Contra Audientor Ito*

*To Ernst and Aurora, my Precious Children*

## Abstract

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Hyperinsulinemic euglycemic clamp, HEC, originally developed for determining insulin-mediated glucose disposal and insulin sensitivity in humans, was used and evaluated in cattle. In the present study the following investigations were performed: the bovine physiological responses during drug-induced insulin resistance, the influence of liberal or restricted concentrate feeding on insulin sensitivity, the impact of two different physical activity levels on insulin sensitivity and muscle characteristics, and finally the expression of abomasal efflux rates in relation to different blood glucose levels.

Clenbuterol and flumethasone treatment induced insulin resistance in calves lasting less than 17 hrs and 3 days post-treatment, respectively. The effect of flumethasone was more manifest than that of clenbuterol.

Insulin sensitivity and muscle characteristics did not differ between one group of calves exposed to light exercise and another group of calves not exercised for 4 to 5 weeks. The results indicated that growth may have an influence on insulin sensitivity.

The blood glucose levels of heifers fed concentrate at the upper limit, according to Swedish feeding standards, were higher than in heifers fed quantities at the lower limit. There was no change in insulin sensitivity or insulin levels between the two groups.

A high plasma glucose level was shown to reduce the abomasal efflux rate and increase the abomasal pH in non-pregnant, non-lactating cows.

The results show that the HEC technique may be a useful means for investigating drug-induced insulin resistance and can also be used to create specific experimental conditions. Ruminant gluconeogenesis may still occur to some extent during HEC application, especially in lactating cows, and therefore this must be considered in future studies.

*Keywords:* insulin, glucose, insulin resistance, muscle characteristics, cattle.

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# Appendix

## Papers I-V

The present thesis is based on the following papers, which will be referred to by roman numerals.

- I. Sternbauer, K., Luthman, J., Hänni, A. and Jacobsson, SO. (1998) Clenbuterol-induced insulin resistance in calves measured by hyperinsulinemic euglycemic clamp technique. *Acta Veterinaria Scandinavica* 39, 281-289.
- II. Sternbauer, K. and Luthman, J. (1998) Flumethasone-induced insulin resistance in calves. *Journal of Veterinary Medicine, Series A* 45, 441-443.
- III. Sternbauer, K. and Essén-Gustavsson, B. (2002) Insulin sensitivity and muscle characteristics in calves at different levels of physical activity. *Journal of Veterinary Medicine, Series A* 49, 449-454.
- IV. Sternbauer, K. and Luthman, J. (2002) Insulin sensitivity in heifers on different diets. *Acta Veterinaria Scandinavica* 43, 107-114.
- V. Holtenius, K., Sternbauer, K. and Holtenius, P. (2000) The effect of the plasma glucose level on the abomasal function in dairy cows. *Journal of Animal Science* 78, 1930-1935.

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Frontcover photo:

©Karin Sternbauer. Faroese calves summer-grazing in front of Tindhólmur on lands of Bø, Vágur.

## Abbreviations

CS	Citrate synthase
BW	Bodyweight
$\beta_2$ -RA	$\beta_2$ -receptor-agonist/s
DA	Displaced abomasum/abomasa
FFA	Free fatty acids
GH	Growth hormone
HEC	Hyperinsulinemic euglycemic clamp
HK	Hexokinase
IM	Intramuscularly
IV	Intravenously
IVGTT	Intravenous glucose tolerance test
LDA	Left displaced abomasum
LDH	Lactate dehydrogenase
M-value	Mean glucose disposal rate per kg live bodyweight
M/I	Insulin sensitivity index
RIA	Radio-immuno assay
SRB	Swedish Red and White breed
SLB	Swedish Holstein breed

# Introduction

## Glucose metabolism and insulin action in ruminants

The newborn calf is very sensitive to insulin. From the time of birth till weaning ends, physical development of the forestomachs occurs along with metabolic maturation due to the change from milk to a forage based diet. The adult ruminant is much less sensitive to insulin and has a lower insulin level than humans and other monogastric species (Janes *et al.*, 1985; Withers, 1992). Most tissues, except the brain and udder, are sensitive to the action of insulin (Hove, 1978; Laarveld *et al.*, 1981; Debras *et al.*, 1989). This is particularly important in lactating animals, where the tissue sensitivity to insulin is of importance for the partition of nutrients between the udder and other tissues.

Although almost all ingested glucose ferments in the rumen of adult ruminants (Kronfeld, 1964), these cannot survive without glucose. Glucose is reduced to lactose in the mammary gland and is a major component of milk. Cows that are prolific or heavy milkers must be able to produce sufficient glucose during lactation, largely by gluconeogenesis in the liver. The hepatic gluconeogenesis mainly utilizes the volatile fatty acid propionate from the rumen fermentation process and glucogenic amino acids such as alanine, to synthesize glucose. In ruminants, the gluconeogenesis occurs during both feeding and fasting, in contrast to monogastric species where it ceases during the post absorptive state due to hepatic glucose absorption (Ballard *et al.*, 1969). However, postprandial changes in glucose and insulin levels also occur in ruminants (Hove & Blom, 1971; Bines *et al.*, 1983), though the response is smaller than in humans (Withers, 1992).

The blood insulin and glucose concentrations are influenced by feeding (Hove & Blom, 1973; Bines *et al.*, 1983; Sano *et al.*, 1990), gender, and age (Röpke *et al.*, 1994). The daytime insulin level is lowest before the morning feed (Hove & Blom, 1971; Bines *et al.*, 1983). Furthermore, the plasma insulin level declines before parturition and is lower during lactation than during the dry period (Vasiliatos & Wangness, 1981; Bines *et al.*, 1983; Holtenius *et al.*, 1993). There has been no report on the seasonal pattern of insulin secretion in domestic cattle, such as seen in red deer stags (McMahon *et al.*, 1997).

Bovine insulin (0.03891mg=1U) differs by three amino acids in comparison with human insulin (0.03846mg=1U) and cross-reactions do occur (Hadley, 1992; Parfitt, 1999). It is synthesized in the  $\beta$ -cells within the pancreatic islets of Langerhans. Circulating insulin molecules are extracted by the liver and kidneys (Hadley, 1992). The effects of insulin are well known and are generally anabolic due to stimulation of protein, lipid and glycogen synthesis. Insulin reduces ketogenesis and gluconeogenesis in the liver but stimulates muscle tissue uptake of glucose, amino acids and ketone bodies. In humans, fat tissues respond to insulin by increasing their uptake of glucose and free fatty acids and by depositing triglycerides. In ruminants, acetate is the main precursor of lipogenesis and its uptake is stimulated by insulin.

## Insulin sensitivity

The measure 'insulin sensitivity' denotes the ability of the peripheral tissues to respond to exogenous insulin (DeFronzo *et al.*, 1979). Various approaches have been used to determine both insulin sensitivity and the degree of insulin resistance. A common test is the intravenous glucose tolerance test, IVGTT, used in humans as well as in cattle (Van Meirhaege *et al.*, 1988; Alzaid & Rizza, 1993; Opsomer *et al.*, 1999; Holtenius *et al.*, 2003). A glucose load is infused i.v. and the subsequent blood insulin response is measured. The hyperglycemic clamp test can be regarded as a further development of the 'glucose tolerance test' and has also been used in cattle by others (Andres *et al.*, 1965; Sano *et al.*, 1991; Hostettler-Allen *et al.*, 1993).

The hyperinsulinemic euglycemic clamp technique (HEC) has become the 'gold standard' method for assessing insulin sensitivity in man (Alzaid & Rizza, 1993). The technique facilitates the standardizing of experimental conditions. This improved method consists of repeated measurement of both insulin and glucose, which strengthens the validity of the findings and reduces the risk of counter-regulatory hormones influencing the results. Endogenous insulin secretion and glucose production are suppressed by a continuous exogenous insulin infusion, while blood glucose concentration is maintained at normal physiological level by regulating the glucose infusion rate. The latter during steady state is used to calculate the insulin-mediated glucose disposal, M-value ( $\mu\text{mol/kg live b.w./min}$ ). The M-value is divided by the mean insulin concentration during steady state, for use as the measure of insulin sensitivity (DeFronzo *et al.*, 1979). In this thesis, the HEC technique was evaluated as a means to expand our knowledge and understanding of ruminant carbohydrate metabolism. Studies were therefore performed under various experimental conditions known to influence glucose metabolism as described below.

### *Drugs and insulin resistance*

It was known before the study was initiated that some drugs, such as  $\beta_2$ -receptor-agonists,  $\beta_2$ -RA, and glucocorticoids, elicit insulin resistance in cattle (Blum & Flueckiger, 1988; Zimmerli & Blum, 1990). The definition of insulin resistance according to Kahn (1978) is: "Insulin resistance exists when normal concentrations of insulin produce a less than normal response" (p. 1893).

The  $\beta_2$ -RA clenbuterol, is well known as an illegal growth promoter in some countries, as it stimulates muscle growth and counteracts fat accretion. In Sweden, its use is forbidden in cattle husbandry, except when used locally to inhibit uterine contractions as an aid in dystocia treatment of mares and heifers/cows (Anon., 2003; Anon., 2004b). The immediate and long-term effects of clenbuterol were reviewed by Beerman (2002). The immediate effects of systemically administered  $\beta$ -agonists include increased heart rate and blood flow. Repeated treatment causes muscle fibre hypertrophy (especially type II fibres) changes in type frequency and altered protein accretion. Down-regulation and desensitization of  $\beta_2$ -receptors are two factors that also have been attributed to long-term use of the drug.

The metabolic effects of glucocorticoid drugs are more obscure. Glucocorticoids are commonly used in cattle farming, where one indication for its use is ketosis. The drug increases the conversion of amino acids to glucose and restricts peripheral glucose utilization (McDonald, 1988). Dexamethasone-induced insulin resistance in Man is reportedly related to both reduced whole-body insulin-dependent glucose oxidation and to non-oxidative glucose disposal and that these changes are not eliminated by co-treatment with ephedrine sulphate (Tappy *et al.*, 1994). It can not be ruled out that the mechanisms underlying drug-induced insulin resistance caused by glucocorticoids and  $\beta$ 2-RA may have common features.

### *Diet and physical activity*

Dietary treatment and increased physical activity have been shown to have a beneficial effect on insulin sensitivity in both normal and insulin-resistant human populations (Proietto *et al.*, 1999; Borghouts & Keizer, 2000).

Increased physical activity level in humans, whether healthy or suffering from non-insulin-dependent diabetes mellitus, has been shown to intensify insulin sensitivity. The post-exercise glucose uptake in muscles is characterized by two phases. The first is an acute, short-term, contraction-induced, non-insulin-dependent phase, lasting for about 2 hours. The second phase is insulin mediated and can last for at least 16 hours, maybe longer (Borghouts & Keizer, 2000). Many cattle farms restrict the animals' physical activity. Our intention of this study was to examine whether the HEC method could detect a difference between two groups of calves, one group with restricted activity and the other allowed light regular exercise for some weeks. Muscle characteristics, such as fibre type composition and oxidative capacity, are also known to be associated with changes in insulin sensitivity in Man (Krotkiewski, 1994). To gain further knowledge in cattle, muscle biopsies were studied at the beginning and end of the trial period.

With respect to the fact that ruminants are considered less sensitive to insulin than monogastric species (Withers, 1992), and the fact that feeding influence bovine glucose and insulin levels (Hostettler-Allen *et al.*, 1993; Röpke *et al.*, 1994), it was also of interest to study whether HEC could detect differences in insulin and glucose response in relation to different diets.

### *Different glucose levels and abomasal efflux rate*

Metabolic disturbances in cattle are most common during the post-partum period. Left-sided displaced abomasum (LDA) is a metabolic disease considered to be preceded by gas accumulation causing the organ to dilate and dislocate (Geishauser, 1995; Van Winden & Kuiper, 2003). LDA accounts for less than 0.5% of all diagnosed diseases in cattle (Anon., 2004a). LDA is often preceded by or accompanied by ketosis (Geishauser, 1995). According to studies in humans, the glucose level is related to the gastric emptying rate (Abrahamsson, 1995; Schvarcz, 1996; Schvarcz *et al.*, 1997). It was therefore of interest to study bovine abomasal efflux rates in relation to low, normal, and high glucose levels, as it has been suggested that insulin resistance could be a factor predisposing to LDA in cattle (Van Meirhaege *et al.*, 1988).

## **Aims of the Thesis**

The overall purpose of the thesis was to evaluate the use of the HEC technique under various metabolic conditions in cattle.

The specific aims:

- to study experimental, drug-induced insulin resistance by HEC testing in calves;
- to study the influence of light physical activity on insulin sensitivity and muscle metabolic characteristics in calves;
- to compare the influence of adding larger and smaller amounts of concentrates to the feed on insulin sensitivity in heifers; and
- to study the influence of high, normal and low glucose levels on abomasal fluid efflux rate in non-pregnant, non-lactating cows.

## Materials and Methods

All experiments were approved by the Ethical Committee for Animal Experimentation, Uppsala.

Drug-induced insulin resistance was studied in Papers I and II, the influence of light physical activity on insulin sensitivity and muscle characteristics in Paper III, the influence of liberal or restricted concentrate feeding on insulin sensitivity in Paper IV and, in Paper V, the influence of different glucose levels on abomasal efflux rate in cows.

### Study designs

Papers I and II described cross-over trials. Papers III and IV reported a standard trial with a parallel group. Paper V had a latin square design, where two treatments were performed one week apart to avoid confounding effects of the first. The HEC technique was used to determine hypo-, normo- and hyperglycemia levels.

### Animals

SRB and SLB, the two most common dairy breeds in Sweden were used. Table 1 presents an overview of the experimental animals at the start of the experiments.

	Study no:				
	I	II	III	IV	V
	Clenbuterol and insulin resistance	Flumethasone and insulin resistance	Exercise and insulin sensitivity	Feeding and insulin sensitivity	Abomasal efflux rate and glucose level
Number	5	5	10	10	4
Breed	SRB + SLB	SRB	SLB	SRB	SRB
Sex	Male calves	Male calves	Male calves	Heifers	Cows
Age (months)	2-4	3-3.5	3-5	3-5.5	3-5 years

Table 1. Overview of animals used with respect to number, breed, sex and age (range).

Calves were housed in single pens either 2.5 m<sup>2</sup> or 2.7 m<sup>2</sup> in size. Cows in study V were tie-stalled.

### Drugs used

In Paper I, clenbuterol (1 µg/kg live b.w.) was administered at 25-26 and 16-17 hours to calves before HEC in series A. In series B, with the same animals 2 months later, the drug was administered 16-17 and 5-6 hours before HEC. In Paper II, flumethasone was used to evaluate the effect of a therapeutic dose (0.5 mg/100 kg live b.w. i.m.) on insulin sensitivity in calves, 24 and 72 hrs post-injection.

## **Exercise and muscle biopsies**

In Paper III, 10 calves were housed in single pens. Of these, 5 were allowed light physical activity on a treadmill (speed 0.6 to 1.5 m/s). The total extent of activity, expressed as distance walked, varied between 8,700 and 12,500 m. The exercise (5 to 15 min.) was performed regularly three times a week for 4 to 5 weeks. HEC tests and muscle biopsies were performed on all calves before and after the experimental period. Samples of muscle were obtained with the biopsy technique (Lindholm & Piehl, 1974) from *m. gluteus medius* under local anesthesia, immediately frozen in liquid nitrogen and stored at -80°C. These procedures were performed 2 to 6 days after the last spell of exercise and analyses of samples were run after all experiments were completed.

## **Feeding**

Feeding plans in all studies were based on common Swedish standards (Spörndly, 1995). In Paper IV, the influence of feeding different proportions of concentrate on insulin sensitivity was studied in young heifers. The groups were fed 0.5 and 2.2 kg concentrate per animal and day. Both groups had free access to hay. The study period was 5 weeks.

## **Technical and analytical data**

The infusions in the study were given by means of a 3-channel-pump (IVAC Medsystem 2860, IVAC Scandinavia AB, Täby, Sweden) except for study V, where a separate syringe pump was used for the insulin infusion (Harvard Apparatus Model 22, Southnatick, MA). Human biosynthetic insulin (Actrapid 100 IU/ml, Novo nordisk, Pharma AB Malmö, Sweden) with a rapid onset of effect was used in all studies. Glucose concentrations used were 100 mg/ml in Studies I-IV), and in Study V, 100 or 500 mg/ml (glucose 100 mg/ml, Kabi Pharmacia, Uppsala, Sweden). In all HEC tests, blood samples were collected in either sodium-heparinized tubes or in serum tubes via a catheter, inserted permanently in the jugular vein, equipped with a three-way stopcock. During the weekly collection of blood in Study IV, a vacutainer system was used (Venoject, Terumo Europe, Belgium). Heparinized blood for the analysis of plasma insulin and cortisol was stored on ice until centrifugation. The separated plasma was frozen immediately after separation and stored at -18°C until analysed. Plasma insulin was measured by RIA (Pharmacia RIA 100, Pharmacia Diagnostics, Uppsala). Serial dilutions of bovine plasma were found to produce displacement curves parallel to the standard curve. Plasma glucose was analysed with the enzymatic glucose oxidase test using a Beckman II glucose analyser (Beckman Instruments, Fullerton, USA). Histochemical staining for myosin ATPase was used to identify the fibre types (Brooke & Kaiser, 1970) in the muscle biopsies. Enzyme activities and glycogen content were measured using fluorimetric methods (Lowry & Passoneau, 1973; Essén *et al.*, 1980; Essén-Gustavsson & Henriksson, 1984).

## Use of HEC technique

The HEC was applied giving a prime insulin infusion dose, varying from 3 mU/kg live b.w./min, decreasing according to an empirically based protocol used in humans (DeFronzo *et al.*, 1979). At 10 minutes, prime solution was changed to a constant insulin dose of 1 mU/kg live b.w./min. The glucose infusion began at 5 min and was adjusted according to the concentration measured in plasma every fifth minute. Plasma collected at 30, 60, 90 and 120 min was obtained for analysis of insulin concentration. The amount of glucose infused was recorded simultaneously. The glucose disposal rate, the M-value, expressed as  $\mu\text{mol/kg/min}$ , was calculated from data obtained during the steady-state period, i.e. 60-120 min of the HEC test. The M-value divided by the average insulin concentration, expressed in mU/l, times 100 was used to calculate the insulin sensitivity index, M/I. In Papers I, II and III, HEC was applied at both the start and end of the experimental period. In Paper IV, HEC was applied at the end of the experimental period only. In Paper V, 4 non-pregnant non-lactating cows were fitted with a permanent abomasal cannula and the efflux rate was measured using a Co-EDTA marker. The cows were randomly exposed to three different plasma glucose concentrations producing, normoglycemia (4.1 mmol/l), hypoglycemia (approx. 2 mmol/l below), and hyperglycemia (approx. 2 mmol/l above), and to a control infusion of physiological sodium chloride (0.9%). The HEC technique was used to establish predetermined steady-state glucose levels.

## Statistics

Data are presented as means and standard deviations ( $\pm\text{SD}$ ). *P*-levels less than 0.05 were considered significant. Student's paired *t*-test was used (StatView, Macintosh) in Studies I and II. In Study III, Wilcoxon signed rank test and Mann Whitney U-test were used to calculate statistical differences within and between groups, respectively. Linear regression was used to analyse relationships between insulin sensitivity, and muscle characteristics (StatView SE Statistical software package for Macintosh). In Study IV, blood chemical data, energy intake and average daily weight gain were analysed using repeated measures analysis of variance (Littell *et al.*, 1991). The general linear models, GLM, procedures of SAS package (SAS® System, 1989), were used. Student's *t*-test for unpaired values was used to compare M values and M/I indexes between low and high fed groups. All data of Study V were analysed using the procedure of mixed models with SAS systems for Windows 6.12 (SAS, 1996).

## Results

The results of the individual papers are presented in numerical order.

### Paper I

Basal plasma glucose and insulin concentrations before clenbuterol treatment were  $4.3 \pm 0.1$  mmol/l and  $3.7 \pm 1.5$  mU/l in series A, when HEC was applied 25-26 hrs post-injection and  $5.1 \pm 0.2$  mmol/L and  $6.0 \pm 1.7$  mU/l in series B, when HEC was applied 16-17 hrs post-injection. In series A, a slight increase in the plasma glucose concentration was noted, but no differences in M or M/I index. In series B, the treatment caused a marked insulin resistance. The glucose infusion rate was remarkably lower during the last hour of infusion. The mean M-value was decreased from 21 to 8  $\mu\text{mol/kg live b.w./min}$ , a 62% reduction of glucose utilization. The M/I index in the insulin resistant calves was 12 compared with 30 in the untreated controls, a 60% reduction.

### Paper II

A single dose of flumethasone increased plasma glucose and insulin levels by 2 mmol/l and 16.5 mU/l, respectively, 24 hours post treatment. The mean M-value was reduced with 74% from 21.6 to 5.5  $\mu\text{mol/kg live b.w./min}$ , while the mean M/I index decreased by 96% from 30.5 to 1.3. At 72 hrs post-flumethasone injection, these effects were abolished except for a persistent 10% increase in plasma glucose concentration.

### Paper III

No differences in insulin sensitivity, plasma insulin or glucose levels were found between (i) the calves exposed to a low level of physical activity regularly for 4-5 weeks and (ii) those not exercised. The metabolic characteristics of muscle were similar in the two groups, both before and after the experimental period. The mean muscle fibre area of types I, IIA and IIB, were significantly increased in both groups at the end of the experimental period. No difference was found between the groups. The mean M-values were 18.9 and 19.1  $\mu\text{mol/kg live b.w./min}$  before and after the experimental period in the exercised group and 17.2 and 14.4  $\mu\text{mol/kg live b.w./min}$  in the control group. The M/I indexes were 30 and 25.5 in the exercised group and 26.6 and 20.5 in the control group, before and after the experimental period. As no differences in insulin sensitivity or muscle characteristics were found between the groups after the experimental period, a linear regression analysis was performed, including all ten calves. At the start of the experiment, the proportion of type I fibres was positively correlated to M/I index ( $r=0.79$ ) and CS activity ( $r=0.66$ ). The CS activity was also found to be negatively correlated to type IIB fibres ( $r=0.83$ ). At the end of experiment, the mean muscle fibre area was negatively correlated to M/I index ( $r=0.67$ ).

## **Paper IV**

Plasma glucose concentration increased in the liberally-fed group, from 4.9 to 5.5 mmol/l at end of the trial. The weekly mean insulin concentration did not differ between groups at any time point and neither did M or M/I index. The mean M-values were 16 and 23  $\mu\text{mol/kg live b.w./min}$  for the liberally and restrictively fed groups, respectively. The mean M/I indexes were 23 and 34, respectively.

## **Paper V**

The results showed no difference in glucose infusion rate between normo- and hyperglycemic treatments ( $p < 0.46$ ). The hypoglycemic treatment required a forceful decrease in glucose infusion rate (10-times less than for normoglycemia) and differed significantly from the other treatments ( $p < 0.001$ ). The mean abomasal efflux rate of hyperinsulinemic hyperglycemic cows was 44% lower vs. the controls. The mean pH in abomasal fluid of hyperglycemic cows was higher (2.3) compared with the other treatments (range 1.9-2.0).

## General discussion

The HEC test, developed for use in humans (DeFronzo *et al.*, 1979), was used after slight modification and evaluated under different experimental conditions in cattle. Earlier studies using the HEC in cattle, differed mainly in one respect – addition of potassium to the infusate (Sano *et al.*, 1991; Sano *et al.*, 1993). In the present study, potassium was not added in any of the experiments. It has been suggested that addition of potassium to the insulin infusion during HEC may affect insulin action (Heinemann *et al.*, 1995). It was concluded from the results of Paper I that insulin *per se* did reduce serum potassium levels, and that addition of potassium seemed unnecessary with respect to the experimental conditions applied.

In Papers I and II the effect of clenbuterol and flumethasone on insulin sensitivity was investigated. The dosages used in both papers were kept within therapeutic limits and based on recommendations in the pharmaceutical literature (Debuf, 1991; Anon., 1994). The drug clenbuterol has earlier been reported to induce a transient insulin resistance in calves (Blum & Flueckiger, 1988; Zimmerli & Blum, 1990). The dosage used in these studies was 25 µg/kg live b.w., compared with only 1 µg/kg live b.w. in Paper I. The HEC test showed clearly the changes in insulin action induced by therapeutic levels of both clenbuterol and flumethasone. It was also observed that the flumethasone-induced insulin resistance was more prominent and longer lasting than that of clenbuterol.

In Man, a high insulin concentration is associated with increased blood flow and it has been shown that a high insulin level also has a vasodilatory effect in skeletal muscle tissue (Raitakari *et al.*, 1995). It has been suggested that glucocorticoids and sympathomimetic agents may impair glucose metabolism by common actions and that withdrawal of the sympathetic tone to the skeletal muscle can be one of these (Paquot *et al.*, 1995).

The  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ratio increased and the insulin sensitivity decreased following clenbuterol treatment in paper I. The increase in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ratio was due to increased serum concentrations of  $\text{Ca}^{2+}$ . In relation to these findings, it becomes interesting to look at studies of antihypertensive drugs in man, of which some has been related to changes in insulin sensitivity. It was shown with the HEC technique, that the angiotensin-converting-enzyme (ACE) inhibitor captopril, had some beneficial effect on insulin sensitivity in man (Berne, 1991). One factor among others that may explaining this action, is drug-induced increase in blood flow of skeletal muscles. Mechanisms regulating skeletal muscle blood flow may therefore be important for determination of the degree of insulin sensitivity. Further, Hänni *et al.* found an inverse relationship between M/I and Ca/Mg ratio, mainly due to the change in Mg concentration (Haenni *et al.*, 1997). Increasing  $\text{Mg}^{2+}$  levels by infusion, has been shown to reduce glucose disposal and decrease basal insulin secretion in Man and lactating sheep ( Zofkova *et al.*, 1988; Gow *et al.* 2003).

During the HEC test in ruminants, hyperinsulinemia could have been expected to reduce calcium concentrations, since it has been shown that a single injection of insulin reduces both plasma calcium and magnesium in sheep (Persson & Luthman, 1974). No reduction in serum ionized calcium or magnesium concentrations of the calves in paper I was observed during the HEC. Metabolic disturbances related to deficiency of magnesium and calcium are common in cattle (paresis puerperalis, hypomagnesemia). Clarifying inter-relationships between calcium, magnesium and insulin sensitivity request further studies.

In Paper III, it was hypothesized that a higher level of physical activity may lead to increased insulin sensitivity, based on studies in humans (Borghouts & Keizer, 2000). A high level of physical activity is an important factor known to increase oxidative capacity and capillary supply in both humans (Henriksson, 1992) and animals (Essén-Gustavsson, 1986). Human studies show that muscle characteristics, and especially increased oxidative capacity and capillary supply, are related to increased insulin sensitivity (Lillioja *et al.*, 1987; Simoneau & Kelley, 1997; Hedman *et al.*, 2000). No increase in insulin sensitivity was measurable in the exercised group after an experimental period of about a month on the predetermined exercise regime. The exercise performed by the calves was light and at this level of activity, it can be argued that no differences in insulin sensitivity were to be expected.

The fact that the calves in paper III were young and increased in body weight and thus muscle mass during the experimental period, must be considered. Fibre areas of all fibre types increased in size, the most marked increase seen in type IIB fibres. The area of type IIB fibres is larger than the area of type I and IIA, and type IIB fibres have the lowest oxidative capacity and capillary supply (Karlström *et al.*, 1994). Fibre areas of the calves in Study III in general, was smaller than in 18-month-old bulls (Essén-Gustavsson & Lindholm, 1990). Comparison of enzyme activity data of the young calves in the present study vis-à-vis the older bulls revealed a higher oxidative capacity indicated by the CS activity. The lower oxidative capacity in the older bulls could be attributable to the relative increase in muscle fibre area and especially of type IIB fibres, whose oxidative capacity is low. As the calves were young, the effect of growth and the increase in muscle fibre areas may have been more important factors during the experimental period influencing on insulin sensitivity, than the light exercise performed.

The influence of feeding on glucose levels has been examined in many studies from different perspectives. The ruminant is adapted to a life on a forage-based diet. The use of different types of concentrates, and concentrate ratios in relation to forage, are factors that have been studied with respect to metabolic agents such as glucose and metabolic hormones (Hove & Blom, 1971; Holtenius *et al.*, 1993; Röpke *et al.*, 1994; Hugi *et al.*, 1997a; Hugi *et al.*, 1997b; Hugi *et al.*, 1998; Holtenius *et al.*, 2003). Blood glucose levels in late gestation cows fed isocaloric diets were shown to increase linearly with increasing content of crude protein, though without a corresponding increase in plasma insulin (Putnam & Varga, 1998). It was therefore expected that the highest nutrition schedule would produce increased blood glucose concentrations in the liberally-fed heifers in Study IV.

The expected increase in blood glucose concentration appeared as early as the 2<sup>nd</sup> week of the trial. No concomitant change in insulin level or insulin sensitivity was observed. In contrast to these results, insulin concentrations were found to increase as a result of feeding a diet rich in concentrates during the fattening period, in bulls, steers and heifers (Röpke *et al.*, 1994). The diets in Paper IV were chosen in accordance with Swedish feeding standards and were not extreme to any degree. It has been shown earlier in cattle that the concentration of propionate in blood is positively correlated to insulin level (Hove & Halse, 1978). It is possible that the composition and contents of different ingredients in the diets would account for this difference in results between the studies, but one should bear in mind that cows in late gestation have naturally low insulin levels (Holtenius *et al.*, 1993).

In the final investigation, the HEC technique was used to produce different glucose levels in blood at a steady-state insulin concentration, in order to estimate the influence on the abomasal efflux rate. It has been found earlier that an infusion of either glucose or glucagon reduced the abomasal fluid turnover time (Holtenius *et al.*, 1998). However, it was not investigated whether this effect was caused by hyperglycemia or a secondary effect induced by elevated insulin level. In the present investigation, the hyperinsulinemic clamp technique was used in combination with concomitant studies on the abomasal fluid rate. The results indicate that hyperinsulinemia as such induces a reduction in the outflow rate.

Since the insulin level must be considered very high (about 800 mU/l) it can not be concluded that the same result will appear at physiological concentrations of insulin. Hyperglycemia *per se*, was found to reduce the abomasal efflux rate and increase abomasal fluid pH. The large differences in glucose infusion rates for normal to high glucose levels, compared with the hypoglycaemic level observed in Paper V, may indicate that the endogenous glucose contribution from the liver gluconeogenesis requires further evaluation. Rose *et al.* (Rose *et al.*, 1997) investigated non-insulin-mediated glucose disposal in lactating cows and reported that it may contribute with up to 80% of total glucose disposed, depending on the considerable conversion of glucose to lactose during lactation. Their findings show that HEC test results in lactating cows should be interpreted with caution.

A transient decrease in insulin sensitivity seems to be a normal physiological response which serves to maintain homeostasis, according to the hypothesis suggested by Bauman and Curie (1980). There are reports where insulin resistance has been associated with cows suffering from LDA (Van Meirhaege *et al.*, 1988; DeCupere *et al.*, 1991) and, as a curiosity fairly recently also in relation to cystic ovarian disease, COD, in cows (Opsomer *et al.*, 1999). In contrast to the studies of Van Meirhaege *et al.* and DeCupere *et al.* above, cows that suffers from LDA have been shown to exhibit low insulin and glucose concentrations prior to developing clinical symptoms (Van Winden *et al.*, 2003). In a recent review of this multifactorial metabolic disturbance, by Van Winden and Kuiper (Van Winden & Kuiper, 2003), it was suggested that the earlier mentioned observed increase in blood glucose levels is secondary to the disease. It could have been overlooked that many of the investigated cows may have been subjected to glucocorticoid treatment, as ketosis is a common co-appearing disease. The finding that a high blood glucose concentration restricts the abomasal efflux rate in cows may therefore suggest a new hypothesis to be tested.

Can it be possible that the observed insulin resistance in cows suffering from LDA is a contributing factor to the pathogenesis of the disease? If so, might some cases of LDA be iatrogenic, caused by veterinary treatment of ketosis by using glucocorticoids?

## General conclusions

Drug-induced insulin resistance of short duration appears not to be harmful to calves. The study showed that a single injection of clenbuterol at a therapeutic level induces a short-lived (less than 6 hrs) insulin resistance, but less prominent than that induced by a single therapeutic injection of flumethasone, which also lasted longer (less than 3days).

Natural factors such as the physical activity and the feeding levels in the present study did not exhibit any differences in insulin sensitivity. In accordance with earlier studies in cattle, it was found that feed with high concentrate content does increase the plasma glucose level in heifers after only one week.

The HEC technique was found suitable to create a pre-determined internal environment in which to study the influence of different glucose levels on the abomasal efflux rate. It was also found that high plasma glucose concentrations increase the abomasal turnover rate and pH in non-lactating non-pregnant cows. This finding may be of importance for further research in the pathogenesis of LDA.

The HEC technique may be a useful means for further studies on ruminant carbohydrate metabolism. However, the limitations considering the non-insulin-dependent glucose disposal of prolific cows must be considered. Moreover it has not yet been shown in any of the studies using HEC in cattle that hepatic gluconeogenesis is completely depressed by the insulin infusion during the test (Rose *et al.*, 1997). The unique metabolic features of ruminants may limit the HEC technique's value in certain experiments, especially when using lactating cows as study objects, whose hepatic gluconeogenesis rate is high and variation wide. To enhance the value of such studies, determination of the non-insulin-dependent glucose disposal rate, radio labelling technique [6,6-glucose<sup>2</sup>H] can be used in combination with the HEC technique (Rose *et al.*, 1997).

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## Populärvetenskaplig sammanfattning

Avhandlingen består av fem separata studier som tillsammans belyser användbarheten av undersökningsmetoden hyperinsulinemisk euglykemisk clamp, HEC, på nötkreatur. Metoden används inom humanmedicinen för att bestämma insulinkänslighet. Försöksobjektet genomgår ett standardiserat protokoll bestående av en kontinuerlig, konstant insulininfusion under två timmar, som har till syfte att hämma den kroppsegna produktionen av insulin genom den kända "negative feedback-control principle". Samtidigt tillförs glukos i blodet kontinuerligt för att upprätthålla en jämn normal glukosnivå. Förutsättningen för detta är regelmässiga mätningar av plasmaglukos var femte minut. Dessa ligger sedan till grund för ändringar som krävs i infusionshastigheten, för att bibehålla den utvalda glukosnivån i blodet. Under infusionens andra timme är förhållandena mycket stabila och blodprov ger efter analys, värden för beräkningar av glukosförbrukningen och insulinkänsligheten. Hos insulinresistenta individer ses en lägre glukosförbrukning jämfört med normala.

De separata undersökningarna som ingår i avhandlingen har visat att HEC-tekniken går att använda på nötkreatur och att den klart kan visa hur insulinkänsligheten påverkas av de farmakologiska substanserna clenbuterol och flumetason.

Beta-agonisten Clenbuterol är mest känd som illegalt tillväxtbefrämjande medel på nötkreatur utomlands. I Sverige används substansen lagligt på djurslaget häst för dess bronchodilaterande, spasmolytiska samt livmoderrelaxerande egenskaper. Den i försöken använda farmakologiska dosen av ämnet visade sig ha en mycket kortvarig reducering av insulinkänsligheten på omkring sex timmar.

Efter en engångsbehandling med en terapeutisk dos av flumetason, en glukokortikoid, sjönk insulinkänsligheten markant, men var åter normaliserad efter tre dagar. Försökskalvarna i de båda inledande försöken visade inte några tecken på obehag som kunde relateras till behandlingen. En kortvarig sänkning av insulinkänsligheten tycks vara helt ofarlig baserat på resultaten och iakttagelserna av försöksdjuren.

Till de naturliga faktorer som kan påverka insulinkänslighet hos människa, är fysisk aktivitet och diet väl studerade.

Fysisk aktivitet har visat sig öka insulinkänsligheten hos både friska och insulinresistenta människor. För att undersöka detta på nötkreatur gjordes ett försök med två grupper av växande kalvar. Den ena gruppen gavs regelbunden motion under fyra till fem veckor och den andra gruppen motionerades inte alls. Det gick inte att påvisa någon skillnad i insulinkänslighet mellan de båda grupperna. Karaktären på arbetet som de motionerade kalvarna utförde var lågintensivt. Även muskelbiopsier togs för att studera eventuella metaboliska förändringar relaterade till insulinkänsligheten. I detta försök fann vi inga sådana, däremot kunde vi visa att muskelfibrernas ytor ökar i storlek med tillväxten och i de undersökta musklerna var det typ II-fibrer som visade den största förändringen.

Foderstatens inflytande på insulinkänsligheten undersöktes också på två grupper av växande kvigor. Den ena gruppen fick en låg andel koncentrat medan den andra fick en hög giva. De utvalda nivåerna låg inom de normala utfodringsnormerna för svensk kalvuppfödning. Förhöjda glukosvärden kunde uppmätas i den grupp som utfodrats med det energitätare fodret. Inga förändringar i insulinkänslighet konstaterades mellan de båda grupperna.

HEC-metoden kan även användas för att studera hur fysiologiska funktioner påverkas av olika plasmaglukosnivåer. Idisslare har till skillnad från människa ett kontinuerligt tillflöde av näring till löpmagen. Det var därför ett intressant fynd att vätskeutflödet från löpmagen på kor förlångsammades vid hög glukos nivå och löpmagsvätskans pH ökade i likhet med människa. Resultatet kan vara av betydelse för uppkomsten av den metaboliska störningen löpmagsförskjutning hos nötkreatur.

Slutsatsen av avhandlingen är att den använda metoden hyperinsulinemisk euglykemisk clamp fungerade väl som undersökningsmetod av icke-lakterande nötkreatur. Metoden kan vara användbar för framtida studier av metaboliska störningar hos nötkreatur förutsatt att man beaktar att idisslarna, till skillnad från människor, kan ha en avsevärt större endogen icke-insulinberoende glukosproduktion, framförallt gäller detta lakterande kor.