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Alterations in Progesterone Catabolic Enzymes by Insulin

Caleb Owens Lemley

Thesis submitted to the Davis College of Agriculture, Forestry and Consumer Sciences at
West Virginia University in partial fulfillment of the requirements for the degree of

Master of Science
in
Reproductive Physiology

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ABSTRACT

Alterations in Progesterone Catabolic Enzymes by Insulin

Caleb Owens Lemley

The current experiments were conducted to determine if insulin could alter the progesterone catabolic enzymes, cytochrome P450 2C and 3A in vitro, utilizing a mouse hepatocyte cell-line, or in vivo, utilizing the ovariectomized ewe as a model. The objectives of the in vitro work were to determine if a four hour challenge with insulin, glucagon or a combination of both insulin and glucagon would lead to a reduction in the relative abundance of CYP2C39 or CYP3A13 mRNA, as well as a decrease in the enzymatic activities of CYP3A and CYP2C subfamilies. A dose dependent decrease ($P < 0.05$) in CYP2C and CYP3A activity was observed in hepatocytes challenged with increasing concentrations of insulin, while a challenge with glucagon had no effect on cytochrome P450 2C or 3A activity. Cytochrome P450 2C activity was decreased ($P < 0.05$) during exposure of cells to 1.0 nM insulin and 0.1 nM glucagon as well as 0.1 nM insulin and 1.0 nM glucagon. Cells cultured with 1.0 nM insulin and 0.1 nM glucagon showed a trend ($P < 0.10$) for a decrease in CYP3A activity. The relative abundance of CYP2C39 and CYP3A13 mRNA showed no response to insulin, glucagon or a combination of insulin and glucagon exposure. These data support a model in which the known insulin-induced decrease in progesterone catabolism is a result of a reduction in CYP2C and CYP3A activity. The objectives of the in vivo experiment, utilizing ovariectomized ewes, were to determine if supplementing feed with either sodium acetate or sodium propionate altered insulin secretion, hepatic activity of cytochrome P450 2C and 3A, and/or progesterone clearance. Sixteen ovariectomized ewes were fed 3 kg/d for 10 d, a diet consisting of 50% corn silage, 38% triticale haylage, 12% soy bean meal, and 200 mL of 3.5 M sodium acetate (energy control $n = 8$) or 2.0 M sodium propionate (gluconeogenic substrate; $n = 8$). Insulin concentrations were determined directly before feeding and at 15, 30, 60, 90, 120, 180, 240, and 300 min. Progesterone clearance from peripheral circulation was measured by giving a 5 mg injection of progesterone into the left jugular vein and collecting blood via the right jugular vein at 2, 4, 6, 8, 10, 15, 20 and 30 min afterwards. Liver biopsies were taken one hour after feeding and used for determination of cytochrome P450 2C and 3A activities. Insulin concentrations in ewes supplemented with sodium propionate were elevated ($P < 0.05$) at 15, 30 and 60 minutes after feeding compared to the sodium acetate group. Cytochrome P450 2C and 3A activity were decreased ($P \leq 0.05$) 1 h after feeding in the sodium propionate treatment relative to sodium acetate treatment. Clearance of progesterone from the peripheral circulation, one hour after feeding, was similar between treatment groups, as well as the average fractional rate constant of progesterone clearance (k). Elucidating a mechanism to decrease progesterone catabolism, thereby increasing concentrations of progesterone, seems a logical approach to ameliorate high rates of embryonic loss without compromising dry matter intake.

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STATEMENT OF THE PROBLEM

Dairy cow pregnancy rates have declined 30% in the last 50 years, and approximately 80% of the total loss of pregnancy occurs during the embryonic stage, before day 42 (Inskeep and Dailey, 2005). Throughout the United States, which has approximately 9 million dairy cows, the industry is losing almost a billion dollars each year due to low fertility. Starbuck et al. (2004) showed increased pregnancy retention to week 7 of gestation in dairy cows that were classified, during week 5 of gestation, as high progesterone (96%) compared to low progesterone (80%). Several authors have shown that removal of the progesterone source results in abortion of the fetus, which could be prevented by daily progesterone injections (McDonald et al., 1952; Csapo, 1956). Plasma progesterone concentrations may be reduced due to a decrease in production and/or an increase in hepatic catabolism. The two major enzymes involved in progesterone catabolism belong to the cytochrome P450 2C and 3A subfamilies and the major metabolites are 21-hydroxyprogesterone and 6 β -hydroxyprogesterone, respectively (Murray, 1991 and 1992).

Both progesterone production and/or hepatic catabolism of progesterone impact peripheral concentrations of progesterone as well as uterine and embryonic exposure to progesterone. Therefore, a drop in progesterone concentrations, due to low production or the normal high rate of catabolism, would result in high embryonic mortality. Miller et al. (1963) reported a short half-life of 33.8 minutes for progesterone, and Bedford et al. (1972, 1974) reported a metabolic clearance rate (volume of blood cleared per unit time)

of progesterone to be approximately 3.5 to 4.3 L/min. Parr et al. (1993) estimated that 90% of progesterone entering the gut and liver is metabolized by these tissues.

Dean and Stock (1975) suggested an internal control mechanism, during pregnancy, which decreased total hepatic cytochrome P450 activity resulting in elevated concentrations of progesterone. Decreasing catabolism of progesterone by decreasing enzymatic activity could increase the concentration of progesterone without the need for progesterone replacement. Smith et al. (2006) observed a dose-dependent decrease in the fractional rate constant of progesterone decay in a murine hepatocyte cell line cultured in the presence of insulin. Hepatocytes treated with physiological concentrations of insulin and glucagon, similar to a postprandial state (i.e. high insulin), resulted in a decrease in the fractional rate constant of progesterone decay compared to control groups as well as a post-absorptive (i.e. high glucagon) treatment group (Smith et al., 2006).

Several authors have shown that a decrease in liver blood flow, by feeding half of maintenance requirements, increases peripheral progesterone concentrations (Parr et al., 1993; Sangsritavong et al., 2002). This relationship between hepatic catabolism of progesterone and insulin concentrations may reveal a physiological control mechanism, which allows an increase in liver blood flow while minimizing deleterious effects on circulating progesterone concentrations. Our objectives were to determine the effect insulin has on decreasing progesterone catabolic enzymes, thereby increasing peripheral concentrations of progesterone, which could be utilized to decrease the high rates of embryonic mortality observed in livestock.

REVIEW OF LITERATURE

Progesterone and Maintenance of Pregnancy

Adequate concentrations of progesterone are needed to maintain a successful pregnancy. Csapo et al. (1956) proposed the “progesterone block hypothesis” while studying pregnancy in the rabbit. This hypothesis states that progesterone maintains pregnancy by directly blocking labor and that removal of progesterone will initiate parturition (Csapo et al., 1956). Garfield et al. (1977) showed an increased insertion of gap junctions between myometrial smooth muscle cells directly before and proceeding through parturition, allowing for intercellular communication and synchronous contraction of these cells. Progesterone prevented gap junction insertion between myometrial smooth muscle cells in rats. Garfield et al. (1978) found that progesterone supplementation in pregnant (days 16 to 17) ovariectomized rats was needed to prevent the insertion of gap junction proteins between these smooth muscle cells. Gap junction proteins are made up of a hexameric assembly of a group of proteins referred to as connexins. The mRNA for connexin-43 in the rat, sheep and human was found to be elevated near term and peaked during delivery due to a drop in concentrations of progesterone (Orsino et al., 1996).

Several findings suggest that progesterone bound to its nuclear receptor (i.e., genomic actions) will modulate uterine contractility by acting to down-regulate contraction-associated genes (Mesiano, 2007). While studying voltage-dependent calcium channels in the rat myometrium, Tezuka et al. (1995) showed a 6.9-fold increase in mRNA expression of voltage-dependent calcium channels directly before delivery (day 22), which could be blocked by treating with progesterone from day 19 to 22.

Hirsbrunner et al. (2002) conducted an in vitro study on spontaneous myometrial contractility, which showed that cows in estrus (0.5 ± 0.3 ng/ml of progesterone) had an elevated contractility in circular smooth muscle cells of the myometrium compared to cows in diestrus (4.8 ± 2.4 ng/ml of progesterone). These differences in myometrial excitation-contraction are brought about by the genomic actions of progesterone, which lead to a relaxed and quiescent uterus able to maintain a successful pregnancy until progesterone withdrawal (Mesiano, 2007).

Progesterone Biosynthesis

Production of progesterone by the corpus luteum is essential in maintaining pregnancy, and any deficiencies in luteal secretion that cause a reduction in progesterone production, can be associated with an abrupt termination of pregnancy (Gorski et al., 1958 and Miller et al., 1963). The first step in progesterone biosynthesis is the endocytosis of cholesterol rich lipoproteins into luteal cells (Christenson et al., 2003). Once inside the cell, cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane via steroidogenic acute regulatory protein (Stocco et al., 2000). Inside the mitochondria, cholesterol can be converted enzymatically into pregnenolone by P450 side chain cleavage (CYP11A1). Pregnenolone will then be converted into progesterone via 3β -hydroxysteroid dehydrogenase, which is located on the smooth endoplasmic reticulum.

Stormshak et al. (1963) estimated that the sheep corpus luteum, having an average turnover rate of two minutes, produces approximately 15 to 30 mg of progesterone a day. In sheep, the ovarian venous blood contains an average concentration of 1.52, 1.82, and 1.10 μ g/ml of progesterone during the early, mid and late luteal phase, while peripheral

concentrations are significantly less. Similar studies in cattle by Gomes et al. (1963), estimated ovarian vein concentrations of progesterone to be 6.2 µg/ml while the jugular vein contained only 11.6 ng/ml. During mid luteal phase, the corpus luteum contained 85 to 570 µg of progesterone with considerable cow-to-cow variation. In a similar study in cows, Erb et al. (1967) estimated mean progestin concentrations in the ovarian vein as 2.6 µg/ml while jugular venous plasma averaged 26.7 ng/ml. Bridges et al. (2000) determined that 300 mg of progesterone per day would maintain pregnancies in lutectomized beef cows. McDonald et al. (1952) determined that 100 mg of progesterone administered intramuscularly daily was needed to maintain pregnancy in a significant number of lutectomized cattle before gestational day 200. However, limited research has been done on estimating luteal progesterone production per day in the cycling or pregnant dairy cow. By using a two minute turnover rate of progesterone (calculated by Stormshak et al., 1963) from the corpus luteum, as well as average luteal content of progesterone (calculated by Erb et al., 1967) we could estimate that a cow produces 300 to 500 mg per day depending on reproductive state.

Metabolic Clearance of Progesterone

Uterine and embryonic exposure to progesterone has been estimated to be higher than peripheral tissue due to a counter-current exchange system within the utero-ovarian adnexa (Einer-Jensen and Hunter, 2000). Ovarian venous plasma concentrations of progesterone may be two to three orders of magnitude greater than peripheral concentrations, and part of this relationship can be accounted for by dilution with peripheral blood; however, low peripheral concentrations of progesterone may also be due in part to a high rate of progesterone catabolism. Miller and colleagues (1963)

reported a half-life of 33.8 minutes for progesterone in cows. Short and Rowell (1962) estimated a half-life of 7.3 minutes at day 115 of gestation in the ewe and 8.1 minutes just prior to lambing. The normal high rate of catabolism may explain why blood draining the ovary has a very high concentration of progesterone, during the mid luteal phase, compared to jugular plasma progesterone concentrations, blood which has already circulated through the liver and gut regions, where the majority of catabolism takes place.

In sheep, Weems et al. (1989) reported progesterone concentrations in the cranial uterine venous plasma to be 5 ng/ml 9 days postestrus compared with 2 ng/ml in the jugular plasma. This difference in concentration could be normalized after resection of both oviductal veins, causing a drop in uterine venous concentrations of progesterone down to 3 ng/ml (Weems et al., 1989). Weems and colleagues hypothesized a transfer of progesterone from the uterine drainage to uterine arteries due to a progesterone gradient, leading to higher concentrations of progesterone in the uterus compared to peripheral tissues. Ginther (1974) diagramed the vascular system of the reproductive tract, which indicated a close proximity between the uterine-ovarian drainage and the uterine artery. Therefore, a counter-current exchange of progesterone between the utero-ovarian vein and the uterine artery could supply progesterone to the uterus, bypassing peripheral circulation (Einer-Jenson, 2005). However, the contribution of a counter current exchange system in the ovarian adnexa has been estimated to be 0.3 – 2% of total steroids (Einer-Jenson and Hunter, 2000). This is in agreement with the subtle differences Weems and colleagues reported in uterine venous and jugular concentrations of progesterone.

Bedford et al. (1972, 1974) reported metabolic clearance rates (volume of blood cleared per unit time) of progesterone to be approximately 3.5 to 4.3 l/min in sheep. Splanchnic clearance of progesterone could account for 30% of peripherally administered progesterone tracer catabolism, and of the total clearance in these splanchnic organs, 70 – 90% of progesterone was cleared by the liver (Bedford et al., 1974). Bedford and colleagues injected progesterone into the jugular vein, which is not the normal route of drainage for endogenously secreted progesterone and may explain why the liver only accounts for 30% of total progesterone clearance in their studies. Other researchers have reported greater hepatic progesterone clearance by injecting or infusing progesterone into the lower extremities thereby mimicking the normal anatomical entry of progesterone into systemic circulation. Gurpide (1975) estimated a 70% hepatic extraction of progesterone in pregnant ewes, and Parr et al. (1993) determined that 96% of the progesterone entering into the liver and gut region was metabolized by these tissues.

Taking into account that the liver is the primary site of progesterone catabolism, several researchers have correlated liver blood flow, through the hepatic portal vein, and the metabolic clearance rate of progesterone. Parr et al. (1993) showed that a 40% increase in liver blood flow in sheep would double the metabolic clearance rate of progesterone. Studies in the dairy cow by Sangsritavong et al. (2002) determined a positive regression between liver blood flow and metabolic clearance rate of progesterone, the relationship was determined to be $\text{metabolic clearance rate} = 1.38(\text{liver blood flow}) + 0.10$ ($r^2 = 0.92$). However, these experiments compared a half maintenance versus twice maintenance diet, which are not only confounded with dry matter intake, but energy and protein intake as well. A drop in metabolic clearance rate may be due to a

decrease in amino acid availability for protein synthesis, thereby decreasing the production of progesterone catabolic enzymes. These strong correlations between progesterone clearance and liver blood flow strengthen the notion that the primary site of progesterone catabolism is the liver, and that altering liver physiology may turn out to be a rational approach to raising progesterone concentrations during early pregnancy when progesterone is essential.

In addition to the experimental approaches mentioned above, in which energy and dry matter intake were confounded, Smith et al. (2006) demonstrated that anestrus ewes orally gavaged with acetate or propionate (0.146 Mcal) and challenged with 20 mg of progesterone, had different rates of progesterone clearance. These gavages were isocaloric and diets contained equal dry matter, but stimulated markedly different insulin secretion patterns. These authors proposed that elevated plasma insulin concentrations were related to the observed reduction in progesterone catabolism. Smith et al. (2006) also defined an in vitro system for determining fractional rates of decay for progesterone by cultured hepatocytes. These authors determined that higher concentrations of insulin (1 and 10 nM) decreased progesterone decay in culture media.

Cytochrome P450 Superfamily

Cytochrome P450s (EC 1.14.14.1; unspecific monooxygenase) are present in most tissues, but most highly expressed in hepatocytes (P450 enzymes account for 1 to 2% of hepatocytes by weight) and are typically embedded in the smooth endoplasmic reticulum (Ruckpaul and Rein, 1984). They are easily isolated by differential centrifugation, known as a microsomal preparation. Cytochrome P450s function as monooxygenases and are involved in the metabolism of a number of important

endogenous compounds including vitamin D3 activation, catabolism of cholesterol to bile acids, and metabolism of all major classes of steroid hormones (Waxman et al., 1991). Cytochrome P450s are also involved in catabolizing a number of different exogenous compounds such as repaglinide, rifampicin (Bidstrup et al., 2003), nifedipine, mephenytoin (Guengerich et al., 1986) and omeprazole (Li et al., 2005). Cytochrome P450s are not self-sufficient and require NADPH and cytochrome b₅ as cofactors, and cytochrome P450 reductase as a coenzyme, to function as catalytic monooxygenases (Yamazaki et al., 1999). Cytochrome P450 reductase serves as an electron carrier by transferring electrons from NADPH to cytochrome P450s (Yamazaki et al., 1999). Cytochrome b₅ has been found to increase the catalytic efficiency of different cytochrome P450s, however the mechanism behind this increased efficiency has eluded researchers for years.

In humans, the most abundant cytochrome P450 belongs to the 3 family, A subfamily and polypeptide number 4, also known as cytochrome P450 3A4 or CYP3A4. The cytochrome P450 3A subfamily accounts for approximately 30 to 40% of total P450 protein in human livers and the 3 major isoforms that have been discovered are CYP3A4, CYP3A5 and CYP3A7. Yuan et al. (2002) reported that CYP3A4 is the most important catabolic enzyme in the CYP3A subfamily in humans and its activity is generally measured using testosterone, midazolam, nifedipine or erythromycin as specific substrates (predominantly catabolized by CYP3A4 in humans and the CYP3A subfamily of enzymes in other mammalian species). Of particular interest is the hydroxylation of testosterone at the 6 β position, which is accomplished by CYP3A4, CYP3A5, CYP3A7, CYP2C9 and CYP2C19; however CYP3A4 accounts for 90% of 6 β -hydroxytestosterone

formation (Yuan et al., 2002), allowing for a fairly selective measurement of CYP3A4 activity with very low reactivity with the other members of the cytochrome P450 superfamily. The predominant substrates used by the pharmaceutical industry to measure CYP2C19 activity are mephenytoin, omeprazole and diazepam (Yuan et al., 2002). Studies by Li et al. (2005) showed that CYP2C19 and CYP3A4 both catabolize omeprazole and that specific inhibitors of CYP2C19 or CYP3A4 are needed to determine the activities for either one of the isozymes individually.

Cytochrome P450s and Steroid Metabolism

Using 11 different cDNA-expressed human cytochrome P450 isozymes transfected into human hepatoma Hep G2 cells, Waxman et al. (1991) determined the predominant cytochrome P450s that catabolize androstenedione, estradiol, progesterone and testosterone. Androstenedione was predominantly catabolized into 6 β -hydroxyandrostenedione along with 3 other unknown products by CYP3A4, CYP3A3, CYP3A5, CYP4B1 and CYP2C8, in order of highest activity to lowest. Estradiol was predominantly catabolized into 2-hydroxyestradiol by CYP3A4, CYP3A3 and CYP1A2 in order of highest activity to lowest. Progesterone was predominantly catabolized into 6 β -hydroxyprogesterone and 16 α -hydroxyprogesterone along with 2 other unknown products by CYP3A4, CYP3A3, CYP4B1, CYP3A5 and CYP2C8, in order of highest activity to lowest. Testosterone was predominantly catabolized into 6 β -hydroxytestosterone, 2 β -hydroxytestosterone and 15 β -hydroxytestosterone along with 3 other unknown products by CYP3A4, CYP3A3, CYP4B1, CYP3A5 and CYP2C8 in order of highest activity to lowest (Waxman et al., 1991). This study using transfected human cytochrome P450s helped provide information on the extent of CYP3A

conservation amongst mammals. However Waxman and colleagues only transfected 11 different isozymes to determine steroid catabolism, leaving a considerable gap in the involvement of the > 40 other P450 isozymes in steroid catabolism (Estabrook, 1998)

Murray (1992) conducted work on progesterone catabolism in sheep, and found that the most abundant progesterone hydroxylation products in hepatic microsomes were 6 β , 21, 2 α and 16 α . Using antibodies raised against different isozymes of rat cytochrome P450s, Murray (1991; 1992) showed the principal isozymes involved in progesterone catabolism in sheep belonged to the cytochrome P450 2C and 3A subfamilies and the major metabolites were found to be 21-hydroxyprogesterone and 6 β -hydroxyprogesterone, respectively (Murray, 1991; 1992). These enzymes were also shown to catabolize testosterone in a manner similar to that demonstrated by Waxman et al. (1991) adding substantial evidence that cytochrome P450 isozymes are conserved not only amongst monogastrics but in ruminant species as well.

Regulation of Cytochrome P450s by Insulin and Glucagon

Sidhu and Omiecinski (1999) found a decreased expression of CYP3A1 mRNA in rat hepatocytes cultured with 1 μ M insulin and then exposed to phenobarbital, a known CYP3A1 inducer in rats. They also found a lower induction of CYP3A1 protein, due to phenobarbital exposure, in cells challenged with 1 nM insulin or physiological concentrations of insulin for a 24 hour period (Sidhu and Omiecinski, 1999). Several researchers have observed an enhanced expression of cytochrome P450 2E1, 2B, 3A and 4A mRNA in chemically induced diabetic rats, and that insulin administration can lower the expression of these cytochrome P450s to normal (Barnett et al., 1990). At least part of this increased expression of cytochrome P450 2E1, an acetone-inducible isozyme, has

been attributed to hyperketonemia. Woodcroft and Novak (1999) showed that pyridine-mediated induction of CYP2E1 mRNA and protein could be substantially decreased when cells were cultured in the presence of 1 μ M insulin for 96 hours compared to primary cell cultures in the absence of insulin; however, insulin did not alter xenobiotic (pyridine)-mediated expression of CYP2B, CYP3A and CYP4A.

By measuring the activity of cytochrome P450s, Saad et al. (1994) found a dose-dependent decrease in testosterone hydroxylation at the 6 β position when rat hepatocytes were cultured with increasing concentrations of insulin (1, 10 and 100 nM), a reaction that is primarily catalyzed by the CYP3A subfamily with minimal contribution from CYP2C13 (Ryan and Levin, 1990). Formation of 6 β hydroxytestosterone decreased 40% when cells were cultured with 10 nM insulin compared to 1 nM insulin (Saad et al., 1994). Saad et al. (1994) exposed hepatocytes to 1 nM insulin along with 0.1, 1.0, 10.0 and 100.0 nM glucagon to elucidate any responses due to glucagon exposure. Hepatocytes exhibited a dose-dependent decrease in 6 β testosterone hydroxylation with increasing concentrations of glucagon. Formation of 6 β hydroxytestosterone was decreased nearly 20% when cells were cultured in the presence of 1.0 nM glucagon compared to 0.1 nM glucagon (Saad et al., 1994). By comparing the work done by Saad et al. (1994) exposing rat hepatocytes to insulin caused a greater decrease in hydroxylation of testosterone at the 6 β position compared to a challenge with physiological concentrations of glucagon in rat hepatocyte primary cultures.

Cyclic AMP and Percent Oxygen

Sidhu and Omiecinski (1995) showed that primary cultures of rat hepatocytes treated with diterpene, forskolin or glucagon stimulated cAMP production, which was

stable for 4 hours, and returned to basal levels by 24 hours. In examining possible signal transducing pathways, Sidhu and Omiecinski (1995) showed that hepatocytes cultured with increasing concentrations of dibutyryl-cAMP, a cAMP analog which mimics the signaling pathway transduced through an active glucagon receptor complex, caused a dose-dependent decrease in CYP2B1, CYP2B2 and CYP3A1 mRNA expression. Sidhu and Omiecinski (1995) found a greater response in CYP2B1 and CYP3A1 mRNA downregulation when substituting 8-(4-chlorophenylthio)-cAMP for the dibutyryl-cAMP analog, and these responses were found to be substantially different at lower concentrations than what is typically reported.

Berry and Skett (1988) were interested in hormonal regulation of steroid metabolism, via measuring metabolism of androst-4-ene-3, 17-dione, after altering cAMP concentrations. They found an inhibition of steroid catabolism (due to enzymatic activity) at 30 and 60 minutes post-treatment at low levels of cAMP exposure, whereas treatment with higher concentrations of cAMP caused a significant stimulation in steroid catabolism suggesting both an inhibitory and stimulatory role for cAMP in steroid catabolism. Banhegyi et al. (1988) reported cAMP-dependent positive control of gluconeogenesis and cAMP-dependent negative control of cytochrome P450s. They proposed that this relationship could help preserve substrates and endogenous compounds from being catabolized during a time of starvation or fasting, when production of these compounds may be limited. However, in vivo studies have shown that most members of the cytochrome P450 3A subfamily are not down regulated via cAMP and appear to be induced by fasting (Banhegyi et al., 1988; Miller and Yang 1984).

Gebhardt (1992) described heterogeneous expression and induction of metabolic enzymes in the liver, hypothesizing that the heterogeneous expression of cytochrome P450s in hepatocytes may be due to micro-environmental differences within the liver. The functional unit of the liver is a mixture of hepatocytes with similar morphology and high variation in hepatocellular function. This functional unit, described as the liver lobule, liver acinus or sickle zone lobule, consists of periportal cells, intermediate cells and perivenous (pericentral) cells (Katz, 1992). Periportal hepatocytes are rich in oxygen that is supplied via arterial and hepatic portal blood, creating an oxygen gradient extending from the periportal to perivenous hepatic tissue. Perivenous hepatocytes are predominant in gluconeogenesis, β -oxidation and glycogenolysis; however, they play a limited role in oxidative xenobiotic metabolism (Katz, 1992). Saad et al. (1994) investigated mixed function monooxygenase activity in rat hepatocytes exposed to different levels of oxygen. Cells were exposed to oxygen levels that are typically found for periportal or perivenous hepatic tissue, 13% and 4%, respectively. By measuring testosterone hydroxylation activities, Saad et al. (1994) found an increase in production of 16 α and 16 β hydroxytestosterone after phenobarbital induction, primarily catalyzed by CYP2B1, in hepatocytes cultured with 13% oxygen. They also found an increase in 6 β hydroxytestosterone production after phenobarbital induction, primarily catalyzed by the CYP3A subfamily, during exposure of hepatocytes to higher oxygen tensions, similar to periportal tissue.

This study demonstrated that oxygen exposure impacts phenobarbital induction of cytochrome P450 activity, which appears to be predominantly in periportal hepatocytes. Cytochrome P450 activity was similar between hepatocytes cultured under 13% and 4%

oxygen (media lacking phenobarbital); however perivenous hepatocytes do not demonstrate phenobarbital induction (Saad et al., 1994). This heterogeneity of mixed function monooxygenases in the liver appears to be due to an oxygen gradient. However, insulin and glucagon challenges in hepatocytes cultured under similar conditions to periportal or perivenous cells retain the ability to downregulate monooxygenase activity after phenobarbital exposure. Krones et al. (2000) found a homogenous distribution of insulin receptor mRNA in the sickle zone lobule, but the insulin receptor protein was localized to the intermediate and distal perivenous hepatocytes. In rat hepatocyte primary cultures exposed to 16% oxygen (periportal) or 8% oxygen (perivenous), the insulin receptor protein was induced 2-fold after the addition of supraphysiological glucose concentrations. Although both culture types had an equal induction of insulin receptor protein, hepatocytes cultured under perivenous conditions (8% oxygen) had twice the abundance of insulin receptor protein compared to their periportal (16% oxygen) counterparts (Krones et al., 2000).

Krones and colleagues (1998) also described the localization of the glucagon receptor to periportal cells. Glucagon receptor mRNA was expressed in both periportal and perivenous hepatocytes; however, the addition of glucose to culture media caused an increase in glucagon receptor mRNA in periportal hepatocytes, but not perivenous hepatocytes. Katz (1992) reported that glucagon acts mainly in periportal tissue and insulin in perivenous tissue; however the study by Saad et al. (1994) indicates that the induction or suppression of cytochrome P450s due to insulin and glucagon challenges can occur in hepatocytes exposed to either of the principal oxygen tensions.

Volatile Fatty Acids

Alterations in ruminant feedstuffs leading to a difference in volatile fatty acid production and ruminal pH can affect microbial yield and amino acid absorption (Russell and Dombrowski, 1980). Thorlacius and Lodge (1973) compared ruminal pH and absorption coefficients (mol/d) in cattle fed a hay diet versus a concentrate diet. They found that ruminal pH ranged from 5.41 to 6.55 compared to 5.78 to 6.53 in hay diets and concentrate diets, respectively. The absorption rates for acetate, propionate and butyrate were 3 times greater in cattle fed the concentrate diet. The ratios of absorption for acetate, propionate and butyrate were 1:2:4 for both diets studied (Thorlacius and Lodge (1973), indicating that neither diet favored absorption of one volatile fatty acid over another. The principal factor that alters absorption rates for acetate, propionate and butyrate is ruminal pH, which can explain the similar absorption rates in the work conducted by Thorlacius and Lodge (1973) where ruminal pH remained in the same range (5.41 to 6.55) between the two diets. The predicted effect of ruminal pH on volatile fatty acid specific absorption rate (absorption rate per unit rumen surface area and per unit concentration) calculated by Pitt et al. (1996) shows that below pH 5.8 absorption rate increased with volatile fatty acid molecular weight. At a pH of 5.5 the predicted absorption rates were 0.17, 0.30 and 0.37 (mol/d) for acetate, propionate and butyrate, respectively, and increasing the pH caused a reduction in the predicted absorption rates for all three, which is most notable for butyrate (Pitt et al., 1996).

The net portal appearance, the total absorption from the lumen of the gastrointestinal tract minus metabolism by these tissues, for acetate and propionate in lactating dairy cows were calculated to be 1880 and 790 mmol/h and this accounted for

40% of the metabolizable energy intake (Reynolds et al., 1988). Hepatocytes lack the capacity to oxidize acetate (Demigne et al., 1986), and the uptake of acetate by the liver is considered to be for biosynthetic, rather than oxidative purposes (Armentano, 1992); however, acetate production by the liver exceeds utilization, making the liver a net producer of acetate. In contrast, propionate clearance by the liver is approximately 90% leaving 10% to be utilized by extrasplanchnic tissues (Reynolds et al., 1988). In vitro saturation of hepatocytes with propionate is reached at 5 mmol/l; leading researchers to believe that in vivo periportal hepatocytes (exposed to < 2 mmol/l of propionate in portal blood) are functioning well below maximal capacity for propionate metabolism (Armentano, 1992). The capacity for propionate metabolism in hepatocytes can be dramatically altered by energy balance, which is most observable in dairy cows experiencing a negative energy balance and lowered propionate metabolism caused by excessive fat buildup in the liver (Armentano et al., 1991).

Volatile fatty acids produced via fermentation in the rumen of the cow contribute 36 to 80% of the energy absorbed from the gut (Casse et al., 1994). While studying the transition period in dairy cows, Reynolds et al. (2003) found that between days 9 and 11 postpartum, 100% of the net liver glucose production was synthesized from propionate, lactate, alanine, and glycerol (69.2, 19.5, 7.5, and 4.2%, respectively). However, metabolic state and age may play an important part in the substrate utilized for glucose production. During sodium propionate infusion into the mesenteric vein of lactating dairy cows, Casse et al. (1994) estimated contributions of L-lactate, alanine, and propionate to hepatic glucose production as 3, 2, and 94%, respectively. A single oral gavage of sodium acetate or sodium propionate in sheep caused an elevation in

concentrations of acetate or propionate in the hepatic portal vein, which was sustained for 4 hours when compared to sheep not orally gavaged with either acetate or propionate (Smith et al., 2006). In this study only acetate could be detected in the jugular plasma supporting the notion that the liver can convert essentially all portal propionate to glucose (Smith et al., 2006).

Gluconeogenesis and Insulin Secretion

In ruminants, which have a very small net portal appearance of glucose, hepatic gluconeogenesis accounts for a large proportion, if not all, of the circulating glucose (Armentano, 1992). Baird et al. (1980) reported that the three major compounds taken up for anabolism of glucose were propionate, lactate and pyruvate. Propionate is completely exogenous in origin, and is only available from gut absorption, while the glucose precursors lactate and pyruvate can be endogenous or exogenous in origin depending on reproductive and nutritional state (Baird et al., 1980). For example, there is a net uptake of pyruvate from the gut in lactating cows, versus a net output in non-lactating cows. In general, gut output of propionate, hepatic output of glucose and hepatic uptake of lactate are all increased during lactation (Baird et al, 1980).

The two major enzymes involved in hepatic gluconeogenesis are pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Velez and Donkin (2005) reported an increase in pyruvate carboxylase in lactating cows fed half maintenance for 5 days compared to *ab libitum* fed cows, while phosphoenolpyruvate carboxykinase was unaffected by feed restriction. In this experiment, insulin and glucagon concentrations were similar between half maintenance and *ab libitum* fed lactating dairy cows. In contrast, work conducted by Reynolds and Tyrrell (1991) showed an increase in insulin

and glucagon with increasing ME intake. The increase in pyruvate carboxylase, although not due to altered levels of insulin or glucagon, supports increased use of lactate and alanine for glucose synthesis in feed deprived ruminants (Filsell et al., 1969). In monogastrics, administration of exogenous glucagon increases phosphoenolpyruvate carboxykinase mRNA (Christ et al., 1988), while glucagon administration to sheep caused an increase in pyruvate carboxylase activity, a key enzyme in converting lactate to glucose (Brockman and Manns, 1974). Williams et al. (2006) reported that administering glucagon to lactating dairy cows did not alter pyruvate carboxylase or phosphoenolpyruvate carboxykinase activity or protein abundance. Williams et al. (2006) determined similar rates of gluconeogenesis by infusing radiolabelled propionate and lactate in lactating dairy cows given glucagon or vehicle.

Pancreatic secretions differ in ruminants compared to non-ruminants because ruminant species lack a measurable, postprandial, absorptive and post-absorptive state (Harmon, 1992), due in part to ruminal fermentation, which leads to a continuous absorption of fermentation end products, and a semi-constant flow of digesta. In ruminants, volatile fatty acids have a dramatic influence on insulin and glucagon secretion. In sheep, infusions of propionate, butyrate, valerate and isovalerate increased insulin secretion, while an equal molar concentration infusion of acetate/kg BW did not affect insulin secretion (Horino et al., 1968). Bassett (1972) found an increase in insulin and glucagon secretion after equal molar infusions of propionate, butyrate, and valerate. Mineo et al. (1990) found a dose-dependent increase in insulin secretion in sheep infused with increasing concentrations of acetate at supraphysiological levels (321 to 5,000 $\mu\text{mol/kg BW}$); however, physiological concentrations of acetate are considered to play a

limited role in regulating insulin secretion in vivo. In ewes, an oral gavage of sodium propionate elevated peripheral concentrations of insulin at 30 and 60 minutes as well as an elevation in glucagon concentrations at 30, 60 and 120 minutes versus ewes orally gavaged with an isoenergetic amount of sodium acetate (Smith et al., 2006). Another factor that contributes substantially to insulin and glucagon secretion is organic matter or the amount of crude protein in feed. In rats, a high-carbohydrate diet caused a 20% increase in insulin secretion, but a high-protein diet resulted in a two-fold greater hepatic extraction of insulin and glucagon (Demigne et al., 1985). The majority of studies showing no response to insulin and glucagon secretion in ruminants fed high-protein diets are due to a failure to measure hepatic extraction of these hormones, which leads to a decrease in peripheral concentrations (Harmon, 1992).

Diabetes

In streptozotocin-induced diabetic male rats, Leonelli et al. (2007) observed a decrease in plasma progesterone concentrations. Arredondo and Noble (2006) reported that patients with diabetes mellitus had an increased incidence of pregnancy loss, which could be normalized by adequate glycemic control. Other researchers have found evidence of lowered progesterone concentrations in women with diabetes; however, these observations have not been associated with production and/or catabolism of progesterone. Ueshiba et al. (2002) found a higher 17-hydroxyprogesterone to progesterone ratio in type 2 diabetic women compared to age matched non-diabetic controls and these results were assumed to be due to an increased activity in 17-hydroxylase, but could be explained by increased progesterone catabolism. Salonia et al. (2006) found a substantial difference in progesterone concentrations during the luteal phase in type 1 diabetic

patients (3.6 nM progesterone) compared to controls (43.6 nM progesterone). These differences in peripheral concentrations of progesterone can be attributed to either a decrease in production of progesterone and/or an increase in hepatic catabolism.

**ALTERATIONS IN PROGESTERONE CATABOLIC ENZYMES,
CYP2C AND CYP3A, IN A MURINE HEPATOCYTE CELL LINE
CHALLENGED WITH INSULIN AND GLUCAGON**

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Abstract

Several authors have suggested that low concentrations of progesterone contribute to pregnancy loss, an effect that may result from abnormally high progesterone catabolism. In humans, cytochrome P450 2C19 (CYP2C19) and 3A4 (CYP3A4) contribute substantially to progesterone catabolism. Recently, it was demonstrated that increasing concentrations of insulin caused a dose-dependent decrease in progesterone catabolism, in mouse hepatocytes. The objectives of this study were to determine if a four hour challenge with insulin, glucagon or combinations of both insulin and glucagon at different dosages can lead to a reduction in the relative abundance of CYP2C39 or CYP3A13 mRNA (homologous to human CYP2C19 and CYP3A4), as well as a decrease in the enzymatic activities of CYP2C and CYP3A subfamilies in these cells. A dose-dependent decrease ($P < 0.05$) in CYP2C and CYP3A activity was observed in hepatocytes challenged with increasing concentrations of insulin, while a challenge with glucagon did not affect cytochrome P450 activity. Cytochrome P450 2C activity was decreased ($P < 0.05$) during exposure of cells to 1.0 nM insulin and 0.1 nM glucagon as well as to 0.1 nM insulin and 1.0 nM glucagon. Cells cultured with 1.0 nM insulin and 0.1 nM glucagon showed a trend ($P < 0.10$) for a decrease in CYP3A activity. The relative abundance of CYP2C39 and CYP3A13 mRNA showed no response to insulin, glucagon or the combination of insulin and glucagon exposure. These data support a model in which the insulin-induced decrease in progesterone catabolism is a result of reductions in CYP2C and CYP3A activity. Moreover, these data support a model in which the regulation of these progesterone catabolic enzymes is mediated post-transcriptionally.

Introduction

Adequate concentrations of progesterone are required for maintenance of pregnancy. Several authors have shown that removal of the progesterone source results in abortion of the fetus, which could be prevented by daily progesterone injections (1, 2). Both progesterone production and/or hepatic catabolism of progesterone impact peripheral concentrations of progesterone as well as uterine and embryonic exposure to progesterone. Therefore, a drop in progesterone concentrations, due to low production or the normal high rate of catabolism, can result in high embryonic mortality. Miller et al. (3) reported a short half-life of 33.8 minutes for progesterone, and Bedford et al. (4,5) reported the metabolic clearance rate (volume of blood cleared per unit time) of progesterone approximated 3.5 to 4.3 L/min.

Parr et al. (6) estimated that 90% of progesterone entering the gut and liver is metabolized by these tissues. Enzymes responsible for progesterone catabolism are located on the endoplasmic reticulum and belong to the cytochrome P450 superfamily (7, 8). These enzymes convert progesterone into its respective hydroxyprogesterone metabolites in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The cytochrome P450 enzymes responsible for the majority of progesterone catabolism belong to the cytochrome P450 2C (CYP2C) and 3A (CYP3A) subfamilies (7, 8).

Dean and Stock (9) suggested an internal control mechanism, during pregnancy that decreases total hepatic cytochrome P450 activity and elevated concentrations of progesterone. Decreasing the activity of the enzymes responsible for progesterone catabolism could increase the concentration of progesterone without the need for

progesterone replacement. Smith et al. (10) observed a dose-dependent decrease in the fractional rate constant of progesterone decay in a murine hepatocyte cell line cultured in the presence of insulin. Treatment of hepatocytes with physiological concentrations of insulin and glucagon, similar to a postprandial state (i.e. high insulin), decreased the fractional rate constant of progesterone decay compared to control groups as well as a post-absorptive (i.e. high glucagon) treatment group (10). Several authors have shown that a decrease in liver blood flow, by feeding at half of maintenance requirements, increased peripheral progesterone concentrations (6, 11). This relationship between hepatic catabolism of progesterone and insulin concentrations may reveal a physiological control mechanism that allows an increase in liver blood flow while minimizing deleterious effects on circulating progesterone concentrations. This would allow provision of the essential nutrients required for normal fetal development during pregnancy without compromising progesterone concentrations to sub-optimal levels.

A relationship between insulin and cytochrome P450 expression was proposed when Barnett et al. (12) and Shimojo et al. (13) observed enhanced expression of CYP3A during diabetes, which was reversed by insulin replacement. Drug induced increases in CYP2E1 mRNA and protein expression were inhibited in hepatocytes cultured for 96 hours with physiological concentrations of insulin (14). In streptozotocin-induced diabetic rats, Barnett et al. (12) showed an increase in CYP3A1 expression and activity, which was reversed by daily insulin therapy. Utilizing a rat hepatocyte primary culture, Sidhu and Omiecinski (15) showed an inhibition of phenobarbital-induced CYP3A1 mRNA expression after insulin exposure. As a corollary to this, lowered insulin concentrations due to diabetes may cause excessive catabolism of progesterone because

of an enhanced expression of cytochrome P450s responsible for the metabolism of endogenous compounds. The objectives of the current study were to determine if the activities of CYP2C and CYP3A in a murine hepatocyte cell line or the relative abundance of CYP2C39 and CYP3A13 mRNA, after a 4 hour challenge with varying concentrations of insulin, glucagon or combinations of both insulin and glucagon may be responsible for the observed decrease in progesterone clearance.

Materials & Methods

Cell Culture for Enzyme Activity Measurements

A mouse hepatocyte cell line from American Type Culture Collection (Manassas, VA; cell-line FL83B, catalogue number CRL-2390) was cultured in F-12K media (American Type Culture Collection), 10% fetal bovine serum (Thermo Fisher Scientifics, Waltham, MA), and 500 IU penicillin/ml and 500 µg streptomycin/ml (Invitrogen, Carlsbad, CA). Cells (1.5×10^6 cells/flask) were expanded into 175 cm² flasks (VWR International, West Chester, PA) and allowed to reach ~ 60% confluence designated, as 0 hours. At this time, media were aspirated and replaced with fresh media containing 0 (control), 0.1, 1 or 10 nM insulin (Sigma Chemical Co., St. Louis, MO), 0 (control), 0.01, 0.1 or 1 nM glucagon (Sigma Chemical Co.); or a combination of insulin and glucagon (0 and 0 (control), 1.0 and 1.0, 1.0 and 0.1 or 0.1 and 1.0 nM, respectively) along with 5 ng/ml of progesterone (Sigma Chemical Co.) and cultured for an additional four hours. After the four hour incubation cells were harvested using trypsin (Invitrogen), subjected to centrifugation at 300 x g, and resuspended in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4). Cells were treated in duplicate on the same day and then replicated in three separate experiments (n = 6).

Microsomal Preparation

After resuspension of cells in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4), cells were homogenized using a glass small clearance Dounce homogenizer. Homogenized cells were subjected to centrifugation at 6000 x g for 8 min. The pellet was discarded and the supernatant was centrifuged at 100,000 x g for 45 min (16). Microsomal pellets were resuspended in 250 μ l of 100 mM potassium phosphate buffer containing 1 mM EDTA. Microsomal recovery was determined using a cytochrome c reductase (NADPH) assay kit (Sigma Chemical Co.). Cytochrome P450 reductase activity was assessed in homogenized cells (i.e. all sub-cellular parts) and then subsequently in microsomal preparations from the same sample. The recovery efficiency cytochrome P450 reductase in our microsomal preparations was determined as described in the manufacturers protocol, and ranged from 70 to 90%.

Cytochrome P450 Activity Assays

Cytochrome P450 2C activity was measured as the omeprazole-dependent oxidation of NADPH (17). A stock solution of omeprazole (lot 115K1873, Sigma Chemical Co.) was prepared in dimethyl sulfoxide at a concentration of 250 mM, and then diluted with 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4) to reach a final concentration of 2.5 mM omeprazole in each reaction containing substrate. All CYP2C reactions contained 1% dimethyl sulfoxide either with or without omeprazole. A stock solution of NADPH (lot 104K7034, Sigma Chemical Co.) was prepared in potassium phosphate buffer containing 1 mM EDTA (pH 7.4) at a concentration of 667 μ M, which was added to each enzymatic reaction to reach a final concentration of 250 μ M. Due to the reactivity of omeprazole with CYP2C and CYP3A,

a 15 min preincubation with 250 μ M ketoconazole (lot 121H0524, Sigma Chemical Co.) was used to inhibit CYP3A (18) and sufficient inhibition was determined by measuring CYP3A activity, following preincubation with ketoconazole, (Figure 1), described in detail below. Utilizing a serially diluted sample of microsomes to validate the cytochrome P450 2C activity assay, we observed a linear relationship between the rates of omeprazole-dependent NADPH oxidation and the activity of cytochrome P450 reductase (NADPH oxidation = 277.1 (cytochrome P450 reductase) + 0.16; Figure 2A).

Cytochrome P450 3A activity was measured as the nifedipine-dependent oxidation of NADPH (19, 20). A stock solution of nifedipine (lot 115K1285, Sigma Chemical Co.) was prepared in acetone at a concentration of 20 mM, and then diluted with 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4) to reach a final concentration of 200 μ M nifedipine in each enzymatic reaction containing substrate. All reactions measuring CYP3A activity contained 1% acetone either with or without nifedipine. A stock solution of NADPH (lot 104K7034, Sigma Chemical Co.) was prepared in potassium phosphate buffer containing 1 mM EDTA (pH 7.4) at a concentration of 667 μ M, which was added to each reaction to reach a final concentration of 250 μ M. Utilizing a serially diluted sample of microsomes to validate the CYP3A activity assay, we determined the linear relationship between the rates of nifedipine-dependent NADPH oxidation and the activity of cytochrome P450 reductase (NADPH oxidation = 181.4 (cytochrome P450 reductase) + 0.39; Figure 2B).

Enzymatic reactions contained 25 μ l of microsomes, 75 μ l of phosphate-buffered NADPH, and 100 μ l of substrate or potassium phosphate buffer loaded into UV star 96 well plates (PGC Scientifics, San Diego, CA). The oxidation of NADPH was determined

Figure 1: Inhibition of cytochrome P450 3A activity using different concentrations of ketoconazole. After a 15 minute preincubation with the inhibitor, microsomes were assayed for cytochrome P450 3A activity.

Figure 2: Validation of cytochrome P450 2C assay utilizing microsomal dilutions versus oxidation of NADPH per minute (A). Validation of cytochrome P450 3A assay utilizing microsomal dilutions versus oxidation of NADPH per minute (B).

by measuring the decrease in light absorbed at 340 nm (37 °C) for 5 min using a Spectra max Plus plate reader (Molecular Devices, Inc., Sunnyvale, CA). Cytochrome P450 2C and 3A reactions remained linear for 10 minutes after the addition of NADPH.

Cell Culture for Determination of mRNA Expression Utilizing Real Time RT-PCR

The same mouse hepatocyte cell line utilized for the determination of cytochrome P450 enzyme activities was treated as described above. After treatment with insulin, glucagon or a combination of insulin and glucagon for 4 hours, media were aspirated and RNA was extracted with TRIzol LS reagent (Invitrogen) and precipitated with 2-propanol following the manufacturers protocol. Concentrations of RNA in each sample were determined using a Nanodrop ND-1000 spectrophotometer. One µg of RNA was electrophoresed through a 1.5% agarose gel to determine sample purity and for visualization of 28S and 18S rRNA bands.

Real Time RT-PCR was performed as previously described (21). Briefly, samples were diluted to 1 µg RNA/µl and reverse transcribed using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) following the manufacturers protocol. Acidic ribosomal protein (ARP) was used as a reference gene, and CYP2C39 and CYP3A13 were chosen due to their homology with the major progesterone catabolic enzymes in the human, CYP2C19 and CYP3A4. Primers for ARP, CYP2C39 and CYP3A13 (Integrated DNA Technologies, Inc., Coralville, IA) are shown in Table 1. Amplification was optimal at an annealing temperature of 59 °C and efficiencies for ARP, CYP2C39, and CYP3A13 were 1.96, 1.95 and 2.16, respectively (Table 1). The relative abundances of mRNA for CYP2C39 and CYP3A13 were

Table 1: Primer sets used for Real Time RT-PCR.

Gene	Accession no.	Forward primer	Reverse Primer	Efficiency
CYP2C39	NM_010003	cct atg gcc ctg tgt tca ct	cag aaa act cct ccc cat ga	1.96
CYP3A13	NM_007819	aag tac tgg cca gag cct ga	aat gca gtt cct tgg tcc ac	2.16
ARP	NM_007475	caa ccc agc tct gga gaa ac	gtg agg tcc tcc ttg gtg aa	1.95

corrected for PCR efficiency, standardized using ARP and expressed relative to a pooled sample, as described by Costine et al. (21).

Statistical Analysis

The effects of insulin or glucagon on enzymatic activity and mRNA expression of CYP2C and CYP3A were analyzed using linear regression, employing the general linear model of SAS. The effects of a combination of both insulin and glucagon were tested with ANOVA utilizing the GLM procedure of SAS (SAS software version 9.1).

Results

Cytochrome P450 2C and 3A activities in mouse hepatocytes incubated for four hours with varying concentrations of insulin are illustrated in Figures 3A and B, respectively. There was a dose-dependent decrease ($P = 0.029$) in CYP2C activity in response to insulin treatment (CYP2C Activity = $-23.4[\text{Insulin}] + 543.7$; $r^2 = 0.20$). Similarly, there was a dose-dependent decrease ($P = 0.002$) in CYP3A activity in response to insulin treatment (CYP3A Activity = $-11.6[\text{Insulin}] + 145.6$; $r^2 = 0.27$). We found no response to insulin treatment in the relative mRNA abundance for CYP2C39 ($P = 0.165$) and CYP3A13 ($P = 0.844$).

Cytochrome P450 2C and 3A activities in mouse hepatocytes incubated for four hours with varying concentrations of glucagon are illustrated in Figures 4A and B, respectively. There was no observed effect of glucagon on CYP2C ($P = 0.143$) or CYP3A ($P = 0.664$) activity. Increasing concentrations of glucagon had no effect on the relative abundance of CYP2C39 ($P = 0.932$) and CYP3A13 ($P = 0.609$) mRNA.

Figure 3: Cytochrome P450 2C activity in mouse hepatocytes after a four hour treatment with varying concentrations of insulin (**A**). Open bar is at 0 hours, before treatment with insulin and progesterone. Cytochrome P450 3A activity in mouse hepatocytes after a four hour treatment with varying concentrations of insulin (**B**). Open bar is at 0 hours, before treatment with insulin and progesterone. Cytochrome P450 2C and 3A activity is expressed per minute per mUnit of cytochrome P450 reductase activity.

Figure 4: Cytochrome P450 2C activity in mouse hepatocytes after a four hour treatment with varying concentrations of glucagon (**A**). Open bar is at 0 hours, before treatment with glucagon and progesterone. Cytochrome P450 3A activity in mouse hepatocytes after a four hour treatment with varying concentrations of glucagon (**B**). Open bar is a 0 hours, before treatment with glucagon and progesterone.

Cytochrome P450 2C and 3A activities in mouse hepatocytes incubated for four hours in the presence of insulin and glucagon at different concentrations are illustrated in Figures 5A and B, respectively. Cytochrome P450 2C activity was unaffected by treatment with 1.0 nM insulin and 1.0 nM glucagon. Cytochrome P450 2C activity in hepatocytes cultured with 1.0 nM insulin and 0.1 nM glucagon decreased ($P = 0.003$) compared to controls. Hepatocytes cultured with 0.1 nM insulin and 1.0 nM glucagon also showed a decrease ($P = 0.029$) in CYP2C activity. Cytochrome P450 3A activity in hepatocytes cultured with 1.0 nM insulin and 0.1 nM glucagon tended to be lower ($P = 0.098$) than the control groups (no insulin or glucagon), and was decreased ($P = 0.006$) compared to CYP3A activity in hepatocytes cultured with 1.0 nM insulin and 1.0 nM glucagon. The relative abundances of CYP2C39 ($P = 0.440$) and CYP3A13 ($P = 0.440$) mRNA in hepatocytes did not change due to treatment with insulin and glucagon.

Discussion

Mouse hepatocytes treated with insulin showed a dose-dependent decrease in the activities of both CYP2C and CYP3A. These observations strengthen the notion that these enzymes are responsible for a majority of progesterone catabolism, as hepatocytes cultured under the same conditions and concentrations of insulin also exhibited a dose-dependent decrease in progesterone catabolism (10). Regulating progesterone catabolism by decreasing CYP2C and CYP3A activities in response to insulin treatment could increase circulating concentrations of progesterone, thus leading to increased reproductive efficiency. Endogenous compounds such as progesterone as well as xenobiotics (i.e. pyridine, phenobarbital and ciprofibrate) have been known to induce the expression of the cytochrome P450s responsible for their catabolism. Extensive studies

Figure 5: Cytochrome P450 2C activity in mouse hepatocytes after a four hour treatment with varying concentrations of insulin and glucagon (**A**, hatched bars). Open bar is at 0 hours, before treatment with insulin, glucagon and progesterone. Cytochrome P450 3A activity in mouse hepatocytes after a four hour treatment with varying concentrations of insulin and glucagon (**B**, hatched bars). Open bar is a 0 hours, before treatment with insulin, glucagon and progesterone. Letters a and b indicate a difference between treatment groups ($P < 0.05$).

exist on the enzymatic processes carried out by cytochrome P450s as well as the mechanisms behind their induction; however, a limited number of studies have been focused on decreasing these enzymes without the use of toxic inhibitors, reviewed by Hewitt et al. (22).

It is well established that chemically induced or spontaneous diabetes enhances expression of cytochrome P450 2E1, 2B, 3A and 4A, and that insulin administration to streptozotocin-induced diabetic rats lowered the expression of these cytochrome P450s to basal levels (12). The hyperketonemia associated with diabetes is responsible, at least in part, for the increased expression of cytochrome P450 2E1, an acetone-inducible isozyme. Woodcroft and Novak (14) showed that pyridine-mediated induction of CYP2E1 mRNA and protein abundance could be substantially decreased when cells were cultured in the presence of 1 μ M insulin for 96 hours; however, insulin exposure did not affect xenobiotic-mediated expression of CYP2B, CYP3A and CYP4A. Sidhu and Omiecinski (15), focused on CYP3A1 expression rather than the CYP3A subfamily, found a decreased expression of CYP3A1 in rat hepatocytes cultured with 1 μ M insulin and then exposed to phenobarbital, a known CYP3A1 inducer in rats. They also found a lower induction of CYP3A1, due to phenobarbital exposure, in cells exposed to physiological (i.e., 1 nM) concentrations of insulin (15).

Saad et al. (23) found a dose-dependent decrease in testosterone hydroxylation at the 6 β position when rat hepatocytes were cultured with increasing concentrations of insulin (1, 10 and 100 nM), a reaction that is catalyzed primarily by the CYP3A subfamily with minimal contribution from CYP2C13 (24). This is similar to our findings, which show a dose-dependent decrease in CYP3A and CYP2C activities using

0.1, 1 and 10 nM insulin concentrations. Formation of 6 β hydroxytestosterone decreased 40% when cells were cultured in 10 nM insulin compared to 1 nM insulin (23), whereas we found a 20% decrease in CYP2C activity and a 60% decrease in CYP3A activity when cells were cultured in 10 nM compared to 1 nM insulin.

In the current study, CYP2C and CYP3A activities were unchanged in a murine hepatocyte cell line treated with increasing concentrations of glucagon. Saad et al. (23) exposed hepatocytes to 1 nM insulin along with 0.1, 1.0, 10.0 and 100.0 nM glucagon to elucidate any responses due to glucagon exposure. In contrast to our results hepatocytes exhibited a dose dependent decrease in 6 β testosterone hydroxylation with increasing concentrations of glucagon. This may have resulted because glucagon concentrations at 10.0 and 100.0 nM are well beyond physiological concentrations. However, formation of 6 β hydroxytestosterone was decreased nearly 20% when cells were cultured in the presence of 1.0 nM glucagon compared to 0.1 nM glucagon (23). In contrast we found no differences in CYP2C or CYP3A activities after a glucagon challenge. Saad et al. (23) found that exposing rat hepatocytes to insulin caused a greater decrease in hydroxylation of testosterone at the 6 β position compared to a challenge with physiological concentrations of glucagon.

Cytochrome P450 3A activity tended to be lower in cells treated with 1.0 nM insulin and 0.1 nM glucagon compared to control cells. Due to the regulatory actions of insulin and glucagon on a number of cytochrome P450s, Sidhu and Omiecinski (15) suggested that insulin and glucagon should be added to hepatocyte cultures at physiological concentrations when studying drug metabolism in vitro. Saad et al. (23) showed a 20 % decrease in 6 β hydroxytestosterone production in cells cultured with 1

nM insulin and 1 nM glucagon compared to 1 nM insulin and 0.1 nM glucagon. In examining possible signal transducing pathways, Sidhu and Omiecinski (25) showed that hepatocytes cultured with increasing concentrations of dibutyryl-cAMP, a cAMP analog which mimics the signaling pathway transduced through an active glucagon receptor complex, caused a dose-dependent decrease in CYP2B1, CYP2B2 and CYP3A1 mRNA expression. They found a greater response in CYP2B1 and CYP3A1 mRNA downregulation when substituting 8-(4-chlorophenylthio)-cAMP for the dibutyryl-cAMP analog, and these responses were substantially different at lower concentrations than typically reported in the literature.

The findings in previous studies, which report downregulation of cytochrome P450 mRNA after exposure to insulin, glucagon or cAMP analogs were from experiments in which cell cultures were exposed to treatments for 24, 48 or 96 hours (which is at least 20 hours longer than our treatments). Liver exposure to elevated concentrations of insulin and glucagon may be as short as one or two hours per day, which may be enough time to regulate activity while having little to no effect on mRNA. In the current experiment, mouse hepatocytes were challenged with physiological concentrations of insulin, glucagon or insulin and glucagon for four hours, and relative abundances of CYP2C39 and CYP3A13 mRNA did not differ. Berry and Skett (26) were interested in hormonal regulation of steroid metabolism, via measuring metabolism of androst-4-ene-3, 17-dione, after altering cAMP concentrations. They found an inhibition of steroid catabolism (due to decreased enzymatic activity) at 30 and 60 minutes post-treatment at low dosages of cAMP, whereas treatment with higher concentrations of cAMP stimulated steroid catabolism, so they suggested both inhibitory

and stimulatory roles for cAMP in steroid catabolism. Banhegyi et al. (27) reported an inverse relationship between cAMP-dependent positive control of gluconeogenesis and the cAMP-dependent negative control of cytochrome P450s. They proposed that this relationship could help preserve substrates from being catabolized during a time of starvation or fasting. However, *in vivo* studies have shown that most members of the cytochrome P450 3A subfamily are not down regulated via cAMP and appear to be induced by fasting (28, 29).

Regulation of CYP2C and CYP3A via insulin describes a logical system, which could facilitate lowered progesterone catabolism. In streptozotocin-induced diabetic male rats, Leonelli et al. (30) observed a decrease in plasma progesterone concentrations. Arredondo and Noble (31) reported that patients with diabetes mellitus had an increased incidence of pregnancy loss that could be normalized by adequate glycemic control. Other researchers have found evidence of lower progesterone concentrations in women with diabetes; however, these observations have not been associated with rates of production and/or catabolism of progesterone. Ueshiba et al. (32) found a higher ratio of 17-hydroxyprogesterone to progesterone in type 2 diabetic women compared to age matched non-diabetic controls and these results were assumed to be due to an increased activity in 17-hydroxylase, but could be explained by increased progesterone catabolism. Salonia et al. (33) found a substantial difference in progesterone concentrations during the luteal phase in type 1 diabetic patients (3.6 nM progesterone) compared to controls (43.6 nM progesterone). These differences in peripheral concentrations of progesterone can be attributed either to lower progesterone production and/or an increase in its catabolism.

In conclusion, a logical mechanism to decrease progesterone catabolism, via cytochrome P450 down-regulation, in animals with excessive metabolism, could be used to increase concentrations of progesterone due to deficiencies in luteal secretion. Our results show a decrease in activity of the progesterone catabolic enzymes after treatment with physiological concentrations of insulin. Diet may play an essential role in regulating peripheral concentrations of progesterone via insulin and glucagon secretion by regulating its hepatic clearance from the body. Methods that increase peripheral concentrations of progesterone will impact uterine and embryonic exposure to progesterone thereby improving retention of pregnancy.

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**THE DIFFERENTIAL RESPONSE OF THE PROGESTERONE
CATABOLIC ENZYMES, CYTOCHROME P450 2C AND 3A IN
EWES SUPPLEMENTED WITH EITHER SODIUM ACETATE OR
SODIUM PROPIONATE**

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Abstract

Pregnancy rates in dairy cows have steadily declined the last 50 years and several authors have suggested that this is related to low peripheral concentrations of progesterone. The primary organ involved in the catabolism of progesterone is the liver, and cytochrome P450 2C and 3A subfamilies account for a large proportion of this catabolism.

Previously, ewes orally gavaged with propionate, a gluconeogenic substrate, had elevated insulin secretion and reduced progesterone catabolism. A dose-dependent decrease in progesterone catabolism was demonstrated by hepatocytes challenged with increasing concentrations of insulin, as well as a dose-dependent decrease in the activity of the two major cytochrome P450s involved in progesterone catabolism. The objectives of the current experiment were to determine if supplementing feed with either sodium acetate or sodium propionate altered peripheral concentrations of insulin, hepatic activity of cytochrome P450 2C and 3A, and/or progesterone clearance. Sixteen ovariectomized ewes were fed 3 kg/d for 10 d a diet consisting of 50% corn silage, 38% triticale haylage, 12% soybean meal, and 200 mL of 3.5 *M* sodium acetate (energy control *n* = 8) or 2.0 *M* sodium propionate (gluconeogenic substrate; *n* = 8). Insulin concentrations were determined immediately before feeding and at 15, 30, 60, 90, 120, 180, 240, and 300 min after feeding. Progesterone clearance from peripheral circulation was measured by giving a 5 mg injection of progesterone into the left jugular vein and collecting blood via the right jugular vein at 2, 4, 6, 8, 10, 15, 20 and 30 min afterwards. Liver biopsies were taken one hour after feeding for determination of cytochrome P450 2C and 3A activities. Insulin concentrations in ewes supplemented with sodium propionate were elevated at 15, 30 and 60 minutes after feeding compared to the sodium acetate group. Cytochrome

P450 2C and 3A activity were decreased 1 h after feeding in the sodium propionate treated ewes relative to sodium acetate. Clearance of progesterone from the peripheral circulation, one hour after feeding, was similar between treatments, as was the average fractional rate constant of progesterone clearance (k). Elucidating a mechanism to decrease progesterone catabolism, thereby increasing embryonic and uterine exposure to progesterone, seems a logical approach to ameliorate high rates of embryonic loss without compromising DMI.

Key words: progesterone catabolism, sheep, insulin, cytochrome P450

Introduction

Pregnancy rates in dairy cows have declined 30% in the last 50 years, and approximately 80% of the total loss of pregnancy occurs during the embryonic stage, before day 42 (Inskeep and Dailey, 2005). Several authors have suggested that low concentrations of progesterone may lead to an abrupt termination of pregnancy (McDonald et al., 1952; Robinson et al., 1989; Stevenson and Mee 1991). Starbuck et al. (2004) reported increased pregnancy retention in dairy cows that were classified as having high concentrations of progesterone compared to low at day 30. Plasma progesterone concentrations may be reduced due to a decrease in production and/or an increase in hepatic catabolism. The two major enzymes involved in progesterone catabolism belong to the cytochrome P450 2C and 3A subfamilies and the major metabolites are 21-hydroxyprogesterone and 6 β -hydroxyprogesterone, respectively (Murray, 1991 and 1992).

Smith et al. (2006) found that ewes orally gavaged with sodium propionate, a gluconeogenic substrate, had increased serum concentrations of insulin and decreased progesterone clearance compared to ewes orally gavaged with an isocaloric amount of sodium acetate (non-gluconeogenic). To elucidate the mechanisms involved in the observed decrease in progesterone catabolism, the authors utilized a murine hepatocyte cell line. Hepatocytes cultured in the presence of insulin had a dose-dependent reduction in the fractional rate constant for progesterone decay compared to controls (Smith et al., 2006). Therefore, the objectives of the current experiment were to determine if altering the type of energy provided, rather than the amount of energy, by supplementing feed

with sodium acetate or sodium propionate, altered insulin concentrations, hepatic activity of cytochrome P450 2C and 3A, and/or progesterone clearance after feeding.

Materials and Methods

Animals and Feeding

Sixteen ovariectomized ewes were blocked by weight and randomly assigned to experimental treatments. Ewes were housed individually in 3 m x 3 m pens for the experimental period of ten days. Animal care and use was according to a protocol approved by the West Virginia University Animal Care and Use Committee (ACUC no. 04-0604). Ewes had ad libitum access to water and were fed 3 kg/d of a standard ewe ration containing 50% corn silage, 38% triticale haylage and 12% soybean meal (on an as fed basis). The DM content of the mixed ration was 38.4% with 17.6% crude protein and 62.1% TDN (DM basis). Equal portions of the ration (1 kg as fed basis) were offered three times daily at 0600, 1400, and 2200 h. The ewes consumed all of the feed within 30 to 45 min. Ewes were allowed 2 d (d 1 and d 2) to adapt to the individual housing and the new feeding schedule before either propionate or acetate supplementation was introduced. Sodium acetate (lot 085K0003, Sigma Chemical Co., St. Louis, MO) or sodium propionate (lot 035K0039, Sigma Chemical Co.) was then introduced in a step-wise manner with 1/3 treatment provided on d 3, followed by 2/3 of the full treatment on d 4 and finally full treatment for the subsequent days (d 5 through d 10). The treatments consisted of either 0.7 mol sodium acetate or 0.4 mol sodium propionate dissolved in 200 ml of water. These amounts of acetate and propionate were calculated to be isocaloric (0.146 Mcal). The sodium acetate or sodium propionate solutions were mixed into the above ration directly before each feeding.

Sample Collection and Hormone Analysis

On d 9, a jugular catheter was inserted prior to the morning feeding. At 0600 h ewes were fed their respective diets. One h after feeding, at 0700 h, ewes were given 5 mg of progesterone i.v. in the left jugular vein. Blood samples were collected via the right jugular vein at 2, 4, 6, 8, 10, 15, 20 and 30 min after progesterone administration into pre-chilled EDTA containing tubes. Progesterone concentrations from the jugular plasma samples were determined using RIA (Sheffel et al., 1982) with a sensitivity of 100 pg/ml and intra- and interassay CV of 4.6 and 7.6%, respectively. Fractional rate constants of progesterone clearance (k) were determined for each individual ewe utilizing the equation $[P]_t = [P]_0 e^{-kt}$, which describes a first order exponential decay curve.

On d 10 blood was collected via jugular venipuncture directly before feeding (0600 h) and at 15, 30, 60, 90, 120, 180, 240 and 300 min after feeding into pre-chilled EDTA containing tubes. Jugular plasma insulin was measured using an insulin RIA kit (lot TKIN 870, Diagnostic Products Corporation, Los Angeles, CA) with a sensitivity of 0.05 nM and intra- and interassay CVs of 1.4 and 7.8%, respectively. The assay was validated to measure sheep insulin in our laboratory. Briefly, plasma dilutions from two different ewes were assayed for insulin and exhibited parallelism with the standard curve.

Liver biopsies were taken on d 10 following the 60 min blood sampling as described by Navarre and Pugh (2002). The wool was removed from the animals' right side and the skin was scrubbed twice with betadine. After determining the location of the ninth intercostal space, 2% lidocaine hydrochloride (lot 6050804, Columbus Serum, Columbus, OH) was administered as a local anesthetic. The skin was punctured using a scalpel and a 14 gauge x 16 cm biopsy needle (J-118A, Jorgensen Laboratories Inc.,

Loveland, CO) was used to collect the liver samples. Liver samples were submerged in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4), placed on ice, and immediately prepared for determination of cytochrome P450 2C and 3A activities.

Microsomal Preparation

After suspending liver tissues in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4), samples were homogenized using a glass Dounce homogenizer. Homogenized samples were subjected to centrifugation at 10,000 x g for 10 min. The pellet was discarded and the supernatant was centrifuged at 100,000 x g for 60 min (modified from Nelson et al., 2001). The microsomal pellets were resuspended in 500 µl of 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4). Microsomal recovery was determined using a cytochrome c reductase (reduced nicotinamide adenine dinucleotide phosphate; **NADPH**) assay kit (product number CY0100, Sigma Chemical Co.). Cytochrome P450 reductase activity was assessed in homogenized tissue (all sub-cellular parts) and subsequently in microsomal preparations from the same sample. The recovery of cytochrome P450 reductase in our microsomal preparations was determined as described in the manufacturers protocol and ranged from 70 to 90%.

Enzymatic Activity

Cytochrome P450 2C activity was measured as the omeprazole-dependent oxidation of NADPH (modified from Li et al., 2005). A stock solution of omeprazole (lot 115K1873, Sigma Chemical Co.) was prepared in dimethyl sulfoxide at a concentration of 250 mM, and then diluted with 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4) to reach a final concentration of 2.5 mM omeprazole in each enzymatic reaction containing substrate. All cytochrome P450 2C reactions

contained 1% dimethyl sulfoxide either with or without omeprazole. A stock solution of NADPH (lot 104K7034, Sigma Chemical Co.) was prepared in potassium phosphate buffer containing 1 mM EDTA (pH 7.4) at a concentration of 667 μ M, which was added to each enzymatic reaction to reach a final concentration of 250 μ M. Due to the reactivity of omeprazole with cytochrome P450 2C and 3A, a 15 min preincubation with 250 μ M ketoconazole (lot 121H0524, Sigma Chemical Co.) was used to inhibit cytochrome P450 3A (Bidstrup et al., 2003) and sufficient inhibition was determined by measuring cytochrome P450 3A activity with nifedipine, described in detail below. Utilizing a serially diluted sample of liver microsomes to validate the cytochrome P450 2C activity assay, a linear relationship was observed between the rates of omeprazole-dependent oxidation of NADPH versus the activity of cytochrome P450 reductase (NADPH oxidation = 44.77(cytochrome P450 reductase) – 0.04; Figure 6).

Cytochrome P450 3A activity was measured as the nifedipine-dependent oxidation of NADPH (modified from Guengerich et al., 1986 and Bork et al., 1989). A stock solution of nifedipine (lot 115K1285, Sigma Chemical Co.) was prepared in acetone at a concentration of 20 mM, and then diluted with 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4) to reach a final concentration of 200 μ M nifedipine in each enzymatic reaction containing substrate. All enzymatic reactions for cytochrome P450 3A contained 1% acetone either with or without nifedipine. A stock solution of NADPH (lot 104K7034, Sigma Chemical Co.) was prepared in potassium phosphate buffer containing 1 mM EDTA (pH 7.4) at a concentration of 667 μ M, which was added to each enzymatic reaction to reach a final concentration of 250 μ M. Utilizing

Figure 6: Validation of cytochrome P450 2C activity. A liver microsome sample was diluted and the rate of omeprazole-dependent oxidation of NADPH as well as the activity of cytochrome P450 reductase were measured to determine linearity of the assay with respect to enzyme amount.

a serially diluted sample of liver microsomes to validate the cytochrome P450 3A activity assay, a linear relationship was observed between the rates of nifedipine-dependent oxidation of NADPH versus the activity of cytochrome P450 reductase (NADPH oxidation = 6.86(cytochrome P450 reductase) – 0.01; Figure 7).

Enzymatic reactions contained 25 µl of microsomes, 75 µl of phosphate-buffered NADPH, and 100 µl of substrate or potassium phosphate buffer loaded into UV star 96 well plates (PGC Scientifics, Fredrick, MD). The oxidation of NADPH was determined by measuring the amount of light absorbed at 340 nm (37 °C) for 5 min using a Spectra max Plus plate reader (Molecular Devices, Inc., Sunnyvale, CA). Cytochrome P450 2C and 3A reactions remained linear for 10 minutes after the addition of NADPH.

Statistical Analysis

The effects of treatments on cytochrome P450 2C, cytochrome P450 3A and progesterone clearance (k) were tested with ANOVA utilizing the GLM procedure of SAS (SAS software version 9.1). The effect of treatment on insulin concentrations were tested using a repeated measures ANOVA utilizing the mixed procedure of SAS with an autoregressive covariance structure, and means were separated using PDIFF option of the mixed procedure. Least square means and standard error of means are reported.

Statistical significance was declared at $P \leq 0.05$.

Results

Insulin concentrations before feeding (0600 h) were the same for ewes in the acetate and propionate groups (Figure 8). At 15 and 30 min after feeding, ewes supplemented with propionate had elevated insulin concentrations compared to the

Figure 7: Validation of cytochrome P450 3A activity. A liver microsomal sample was diluted and the rate of nifedipine-dependent oxidation of NADPH as well as the activity of cytochrome P450 reductase was measured to determine linearity of the assay with respect to enzyme amount.

Figure 8: Plasma concentrations of insulin in ewes supplemented with 0.7 mol sodium acetate or 0.4 mol sodium propionate at different time points relative to feeding at 0 min. * indicates a difference between treatment groups at that time point ($P < 0.05$).

acetate-supplemented group ($P < 0.05$; Figure 8). At 60 min after feeding, insulin concentrations in propionate-supplemented ewes began to decline, but were still elevated compared to the acetate-supplemented group ($P < 0.05$; Figure 8). During the postprandial state, 90 min to 300 min, insulin concentrations did not differ among or between groups and were the same as time 0 (or 8 h after the previous feeding).

Cytochrome P450 2C activity in liver tissue from the propionate-supplemented ewes was reduced ($P < 0.05$) to nearly one-half of the activity in liver tissue from acetate-supplemented ewes (Figure 9). Cytochrome P450 3A activity was reduced in liver of the propionate-supplemented ewes ($P = 0.05$) to nearly one-half of that in acetate-supplemented ewes (Figure 9).

Progesterone concentrations prior to the injection of 5 mg of progesterone were less than 0.3 ng/ml. In the sodium propionate and sodium acetate supplemented ewes 72% of the injected progesterone, 10 min after progesterone administration, had been either catabolized by the liver or taken up by extra-hepatic tissues (Figure 10). Due to the high variability of progesterone clearance from the peripheral circulation during the first 6 min of blood sampling these time points were not used in determining the fractional rate constants of progesterone clearance. Fractional rate constants of progesterone clearance (k) were determined for each individual ewe utilizing progesterone concentrations from 8, 10, 15, 20 and 30 min relative to progesterone injection. Although the progesterone clearance was numerically lower in the propionate-supplemented group this difference was not significant (Figure 11). A power test, using the means and variances generated from these data, estimated that 40 observations for each treatment would be needed to detect a significant effect of treatment.

Figure 9: Enzymatic activity of cytochrome P450 2C and cytochrome P450 3A in liver biopsies taken 1 hour after feeding in ewes supplemented with sodium acetate or sodium propionate. Enzymatic activities are scaled to cytochrome P450 reductase. * indicates a difference in treatment ($P \leq 0.05$).

Figure 10: Progesterone concentrations in ewes injected with 5 mg of progesterone 1 hour after feeding rations supplemented with 0.7 mol sodium acetate or 0.4 mol sodium propionate. Plasma samples for determination of progesterone concentrations were collected at 2, 4, 6, 8, 10, 15, 20 and 30 min.

Figure 11: Mean fractional rate constants of progesterone decay in ewes supplemented with 0.7 mol sodium acetate or 0.4 mol sodium propionate. Fractional rate constants were determined by graphing progesterone concentrations versus time (8, 10, 15, 20 and 30 min) with the equation $[P]_t = [P]_0 e^{-kt}$.

Discussion

Insulin concentrations peaked 30 min after feeding in both the acetate supplemented and the propionate supplemented groups. The increase in insulin concentration was three times greater in propionate as compared to acetate-supplemented ewes, which would be expected because of the gluconeogenic properties of propionate. Volatile fatty acids produced via fermentation in the rumen of the cow contribute 36 to 80% of the energy absorbed from the gut (Casse et al., 1994). While studying the transition period in dairy cows, Reynolds et al. (2003) found that between days 9 and 11 postpartum, 100% of net liver glucose production was synthesized from propionate, lactate, alanine, and glycerol (69.2, 19.5, 7.5, and 4.2%, respectively). Casse et al. (1994) estimated contributions of L-lactate, alanine, and propionate to hepatic glucose production, during sodium propionate infusion into the mesenteric vein of lactating dairy cows, as 3, 2, and 94%, respectively. These results are in agreement with the present observations of increased jugular concentrations of insulin in ewes supplemented with sodium propionate.

However, metabolic state and age may play an important part in the substrate utilized for glucose production, thereby leading to elevated insulin secretion. In vitro saturation of hepatocytes with propionate is reached at 5 mmol/l. Several authors have suggested that in vivo periportal hepatocytes, exposed to < 2 mmol/l of propionate in portal blood, are functioning well below maximal capacity for propionate metabolism (Armentano, 1992). The capacity for propionate metabolism in hepatocytes can be dramatically altered by energy balance, which is most observable in dairy cows

experiencing a negative energy balance and lowered propionate metabolism caused by excessive fat buildup in the liver (Armentano et al., 1991).

A single oral gavage of sodium acetate or sodium propionate in sheep elevated concentrations of acetate or propionate in the hepatic portal vein, which was sustained for 4 hours when compared to sheep not orally gavaged with either acetate or propionate (Smith et al., 2006). An oral gavage of sodium propionate resulted in greater insulin concentrations in peripheral circulation at 30 and 60 minutes as well as higher glucagon concentrations at 30, 60 and 120 minutes versus animals orally gavaged with sodium acetate (Smith et al., 2006). The peak insulin concentration and the pattern of the insulin response curve to either propionate or acetate observed by Smith et al. (2006) was very similar to the response observed in this study, despite the difference in the method of propionate or acetate delivery between the two studies.

Murray (1992) found that the most abundant hydroxylation products of progesterone in hepatic microsomes of sheep were 6 β , 21, 2 α and 16 α hydroxyprogesterone metabolites. A member of the cytochrome P450 2C subfamily was involved in metabolizing progesterone into its 21-hydroxyprogesterone metabolite, and a member of the cytochrome P450 3A subfamily was involved in metabolizing progesterone into its 6 β -hydroxyprogesterone metabolite, as determined by measuring different metabolites after immunoinhibition with specific anti-P450s (Murray 1991, 1992). Dean and Stock (1975) observed a decrease in overall hepatic microsomal cytochrome P450s during pregnancy, which led them to hypothesize a biological control mechanism to elevate serum progesterone concentrations by decreasing catabolism. In the current experiment, activities for cytochrome P450 2C and 3A decreased 1 hour after

feeding in ewes supplemented with sodium propionate compared to sodium acetate. This time is congruent with the time over which propionate supplementation continued to result in increased secretion of insulin, which would be expected to decrease hepatic progesterone catabolism. Therefore, we believe the decreased progesterone clearance observed by Smith et al. (2006) was mediated by decreased cytochrome P450 2C and 3A activities, although clearance of progesterone from peripheral circulation in the present study did not differ.

Bedford et al. (1972, 1974) reported metabolic clearance rates (volume of blood cleared of progesterone per unit time) for progesterone to be approximately 3.5 to 4.3 L/min in sheep. Splanchnic clearance rate of progesterone could account for 30% of total progesterone metabolism, and 70 – 90% of the total clearance of progesterone in these splanchnic organs was cleared by the liver (Bedford et al., 1974). In these studies metabolic clearance rates were determined by infusing progesterone into the peripheral circulation via the jugular vein, which is different from the *in vivo* source of progesterone, primarily produced in the ovary or the gravid uterus both of which are part of the portal drained viscera. Therefore, others have reported greater hepatic progesterone clearance, by infusing progesterone into the lower extremities because this progesterone is first taken to the liver for metabolism before entering whole body circulation. Gurpide (1975) estimated a 70% hepatic extraction of progesterone in pregnant ewes, and Parr et al. (1993) determined that 96% of the progesterone entering into the liver and gut region was metabolized by these tissues.

Taking into account that the liver is the primary site of progesterone catabolism, several researchers have correlated liver blood flow through the hepatic portal vein and

metabolic clearance rate of progesterone. Parr et al. (1993) showed that a 40% increase in liver blood flow in sheep almost doubled the metabolic clearance rate of progesterone. Studies in the dairy cow by Sangsritavong et al. (2002) determined a positive correlation between liver blood flow and metabolic clearance rate of progesterone. The relationship was determined to be $\text{metabolic clearance rate} = 1.38(\text{liver blood flow}) + 0.10$ ($r^2 = 0.92$). However, these studies compared high versus low maintenance diets, which differed in energy, protein and DMI. This strong correlation between progesterone concentration and liver blood flow only strengthens the notion that the primary site of progesterone catabolism is the liver, and that altering liver physiology may be a plausible approach to raising progesterone concentrations during early stages in pregnancy when progesterone is essential. In the current work, consumption of dietary DM was equal for the two treatment groups, and dietary treatments were both isocaloric and isonitrogenous. These diets resulted in a 45% decrease in the activity of progesterone catabolic enzymes, without confounding changes in DMI or the amount of crude protein available.

After an oral gavage of sodium acetate or sodium propionate, serum progesterone concentrations began to diverge at 30 min in anestrus ewe lambs given an i.m. injection of 20 mg of progesterone and were greater in the sodium propionate ewes at 3 and 4 hours after treatment (Smith et al., 2006). Lower progesterone clearance was then associated with elevated insulin concentrations rather than elevated glucagon concentrations. Utilizing a murine hepatocyte cell line, a dose-dependent decrease in the fractional rate constant of progesterone clearance was observed with increasing concentrations of insulin (Smith et al., 2006). These results are in agreement with work

by Selvaraju et al. (2002), who demonstrated that a s.c. injection with long acting bovine insulin elevated serum progesterone concentrations in repeat breeder cows.

In the current work, cytochrome P450 activity was reduced 45% with propionate supplementation; however we observed no difference in progesterone fractional clearance rates. Administering progesterone into the jugular vein may have limited hepatic catabolism and/or increased extra-hepatic uptake of progesterone from the plasma. In conclusion, ewes supplemented with sodium propionate (a gluconeogenic substrate) had elevated concentrations of insulin at 15, 30, and 60 minutes after feeding as well as lower cytochrome P450 2C activity and a trend for lower cytochrome P450 3A activity compared to ewes supplemented with sodium acetate (energy control).

Implications

Insulin appears to play an essential role in regulating progesterone catabolic enzyme activity in the liver. Identifying feedstuffs that can increase insulin without concomitantly increasing liver blood flow might help mitigate excessive progesterone catabolism without the need to reduce intake below maintenance requirements. Progesterone production and/or hepatic catabolism of progesterone both impact peripheral concentrations of progesterone as well as uterine and embryonic exposure to progesterone. Therefore elucidating a mechanism that decreases the activity of progesterone catabolic enzymes could provide for increased progesterone concentrations and possibly decreased embryonic loss.

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GENERAL DISCUSSION

Exposing a mouse hepatocyte cell line to varying concentrations of insulin, glucagon or a combination of both insulin and glucagon for four hours had an impact on the activity of cytochrome P450 2C and 3A subfamilies, however, these insulin and glucagon challenges did not alter the relative abundance of cytochrome P450 2C39 and 3A13 mRNA. Insulin challenges of 0.0, 0.1, 1.0 and 10.0 caused a dose dependent decrease in cytochrome P450 2C and 3A activities, while glucagon exposure had no affect on cytochrome P450 activity. Cytochrome P450 2C activity was unaffected by treatment with 1.0 nM insulin and 1.0 nM glucagon compared to controls (no insulin or glucagon). Cytochrome P450 2C activity in hepatocytes cultured with 1.0 nM insulin and 0.1 nM glucagon or 0.1 nM insulin and 1.0 nM glucagon decreased compared to controls. Cytochrome P450 3A activity in hepatocytes cultured with 1.0 nM insulin and 0.1 nM glucagon tended to be lower than the control groups, and was decreased compared to CYP3A activity in hepatocytes cultured with 1.0 nM insulin and 1.0 nM glucagon. These observations strengthen the notion that these enzymes are responsible for a majority of progesterone catabolism, as hepatocytes cultured under the same conditions and concentrations of insulin also exhibited a dose-dependent decrease in progesterone catabolism (Smith et al., 2006). The differential response in progesterone catabolic enzyme activity, after insulin and glucagon exposure, leads us to believe that this mechanism may be a plausible approach to decrease progesterone catabolism in vivo.

To test this hypothesis, we set out to determine if supplementing feed with either sodium acetate (energy control) or sodium propionate (gluconeogenic substrate) altered insulin concentrations, hepatic activity of cytochrome P450 2C and 3A, and/or

progesterone clearance. At 15 and 30 min after feeding ewes supplemented with propionate had elevated insulin concentrations compared to the acetate-supplemented group. At 60 min after feeding insulin concentrations in propionate-supplemented ewes began to decline but were still elevated compared to the acetate-supplemented group. At one hour after feeding, when insulin concentrations were still elevated, the activity of cytochrome P450 2C and 3A were decreased in propionate-supplemented ewes compared to acetate-supplemented ewes; however, no difference in the clearance of progesterone from peripheral circulation was detected between the treatment groups. Insulin clearly regulates progesterone catabolic enzymes, and identifying feedstuffs that decrease their activity in vivo, without altering blood flow to the liver, could increase peripheral concentrations of progesterone.

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