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Alterations in Progesterone Catabolic Enzymes, CYP2C and CYP3A, in Hepatocytes Challenged with Insulin and Glucagon

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Abstract: Several authors have suggested that low progesterone concentrations contribute to pregnancy loss, an affect that may result from abnormally high progesterone catabolism. Recently, we demonstrated, *in vitro*, that increasing insulin concentrations caused a dose-dependent decrease in progesterone catabolism by hepatocytes. The objectives of this study were to determine if CYP2C, CYP3A or both enzymes were responsible for the insulin mediated alteration in progesterone catabolism. A dose-dependent decrease 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 2C or 3A activity. As expected, due to the progesterone catabolizing properties of CYP2C and CYP3A, we found elevated concentrations of progesterone in the media of hepatocyte cell cultures that contained insulin, while a challenge with glucagon had no affect on progesterone concentrations. Cytochrome P450 2C activity was decreased 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 for a decrease in CYP3A activity. Cells that had a decrease in the activity of both CYP2C and CYP3A after a challenge with both insulin and glucagon had elevated progesterone concentrations. These data support a model in which the insulin-induced decrease in progesterone catabolism is a result of reductions in both CYP2C and CYP3A activity.

Key words: Cytochrome P450, hepatocytes, insulin, progesterone catabolism

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 (McDonald *et al*., 1952; Csapo, 1956). Both progesterone production and hepatic catabolism of progesterone impact peripheral concentrations of progesterone as well as uterine and embryonic exposure to progesterone. Miller *et al*. (1963) reported a short half-life of 33.8 min for progesterone and Bedford *et al*. (1972, 1974) reported a metabolic clearance rate (volume of blood cleared per unit time) for progesterone of approximately 3.5-4.3 L min^{-1}. Parr *et al*. (1993) estimated that 90% of progesterone entering the gut and liver is metabolized by these tissues. Cytochrome P450 enzymes (1.14 14.1; unspecific monooxygenase) 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 P450 enzymes are also involved in the metabolism of a number of different xenobiotics and in humans the cytochrome P450 3A subfamily is generally regarded as the most important catabolic enzyme involved in drug metabolism (Yuan *et al*., 2002). The cytochrome P450 2C (CYP2C) and 3A (CYP3A) subfamilies (You, 2004) are responsible for converting progesterone into its hydroxyprogesterone metabolites in the presence of reduced nicotinamide adenine dinuleotide phosphate (NADPH) (Sakuma *et al*., 2000; Yamazaki and Shimada, 1997).

Dean and Stock (1975) suggested that during pregnancy an internal control mechanism decreases total hepatic cytochrome P450 activity, elevating concentrations of progesterone. 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. 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

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progesterone decay compared to control groups and a post-absorptive (i.e., high glucagon) treatment group (Smith *et al*., 2006). A relationship between insulin and cytochrome P450 expression was proposed when Barnnett *et al*. (1990) and Shimojo *et al*. (1993) observed enhanced expression of CYP3A during insulin dependent diabetes, which was reversed by insulin replacement. Drug induced increases in CYP2E1 mRNA and protein expression were inhibited in hepatocytes cultured for 96 h with physiological concentrations of insulin (Woodcroft and Novak, 1999). In streptozotocin-induced diabetic rats, Barnnett *et al*. (1990) showed an increase in CYP3A1 expression and activity, which was reversed by daily insulin therapy. Utilizing a rat hepatocyte primary culture, Sidhu and Omiecinski (1999) showed an inhibition of phenobarbital-induced CYP3A1 mRNA expression after insulin exposure. As a corollary to this, lowered insulin concentrations due to insulin dependent 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 previously observed decrease in progesterone clearance was due to a reduction in the activities of CYP2C and/or CYP3A after a 4 h challenge with varying concentrations of insulin, glucagon or combinations of both insulin and glucagon.

MATERIALS AND METHODS

Cell culture: 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^{-1} and 500 μg streptomyocin mL^{-1} (Invitrogen, Carlsbad, CA). Cells (1.5×10^6 cells $flask^{-1}$) were expanded into 175 cm^2 flasks (VWR International, West Chester, PA) and allowed to reach ~60% confluence, designated as 0 h. 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^{-1} of progesterone (Sigma Chemical Co.) and cultured for an additional 4 h. A 1 mL sample was removed from each flask and stored at -20°C for later analysis of progesterone concentrations. After the 4 h incubation cells were harvested using trypsin (Invitrogen), subjected to centrifugation at 300×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 3 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×g for 8 min. The pellet was discarded and the supernatant was centrifuged at 100,000×g for 45 min (Sukhodub and Burchell, 2005). Microsomal pellets were resuspended in 250 μL of 100 mM potassium phosphate buffer containing 1 mM EDTA. Microsomal recovery was determined using a cytochromec reductase (NADPH) assay kit, as described in the manufacturers protocol (product number CY0100, Sigma Chemical Co.). Briefly, cytochrome P450 reductase activity was assessed in homogenized tissue (all sub-cellular parts) and subsequently in microsomal preparations from the same sample and used to standardize cytochrome P450 2C and 3A activity in all subsequent samples. The recovery of cytochrome P450 reductase ranged from 70-90%.

Cytochrome P450 activity assays: Cytochrome P450 2C activity was measured as the non-ketoconazole-inhibitable, 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 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 μM, which was added to each enzymatic reaction to reach a final concentration of 250 μM. Due to the reactivity of omeprazole with CYP2C and CYP3A, a 15 min preincubation with 250 μM ketoconazole (lot 121H0524, Sigma Chemical Co.) was used to inhibit CYP3A (Bidstrup *et al*., 2003) and sufficient inhibition was determined by measuring CYP3A activity, following the 15 min preincubation with ketoconazole (CYP3A activity = -0.96 (ketoconazole) + 229.08; $r^2 = 0.88$), 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; $r^2 = 0.97$).

Cytochrome P450 3A activity was measured as the nifedipine-dependent oxidation of NADPH, modified from (Guengerich *et al*., 1986; 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 μM 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 μM, which was added to each reaction to reach a final concentration of 250 μM. 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; $r^2 = 0.94$).

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, San Diego, CA). The oxidation of NADPH was determined 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 min after the addition of NADPH.

Progesterone assay: One mL of culture media was sampled from the 0 and 4 h treatment groups and stored at -20°C until analysis progesterone. Progesterone concentrations in cell culture media were determined using RIA (Sheffel *et al*., 1982) with a sensitivity of 100 pg mL^{-1} and intra- and interassay CV of 3.5 and 6.2%, respectively.

Statistical analysis: The effects of insulin or glucagon on enzymatic activity 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 on CYP2C and CYP3A activity were tested with ANOVA utilizing the GLM procedure of SAS. The effects of insulin, glucagon or a combination of both insulin and glucagon on progesterone concentrations in cell culture media 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 4 h with varying concentrations of insulin are illustrated in Fig. 1a and b, respectively. There was a dose-dependent decrease ($p<0.05$) in CYP2C activity in response to insulin treatment (CYP2C Activity = -23.4 (Insulin) + 543.7; $r^2 = 0.20$). Similarly, there was a dose-dependent decrease ($p<0.01$) in CYP3A activity in response to insulin treatment (CYP3A Activity = -11.6 (Insulin) + 145.6; $r^2 = 0.27$). After a 4 h incubation with progesterone (5 ng mL^{-1})

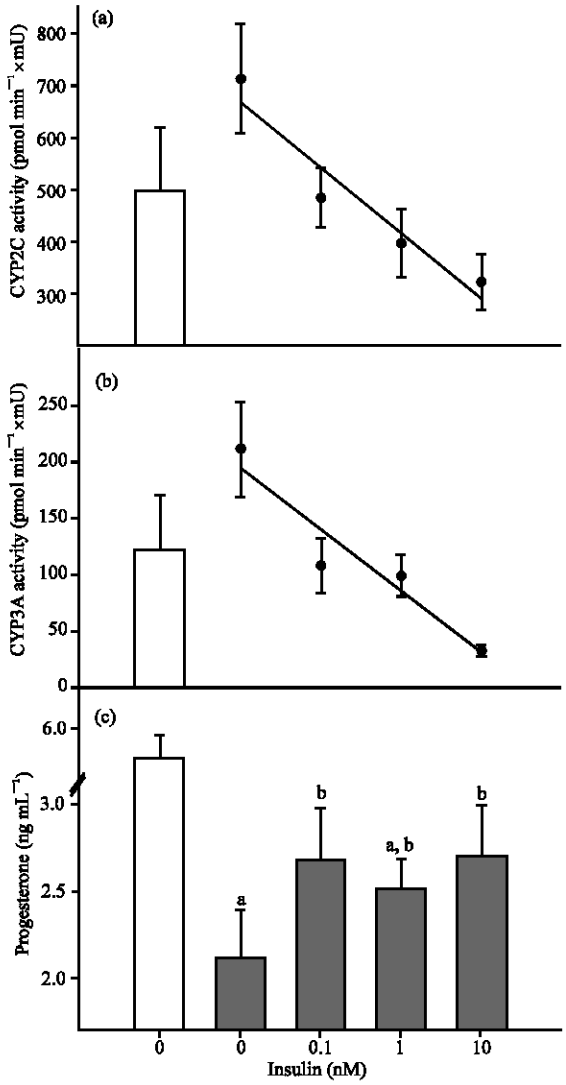

Fig. 1: Cytochrome P450 2C activity, 3A activity and progesterone concentrations in a mouse hepatocyte cell-line challenged with increasing concentrations of insulin

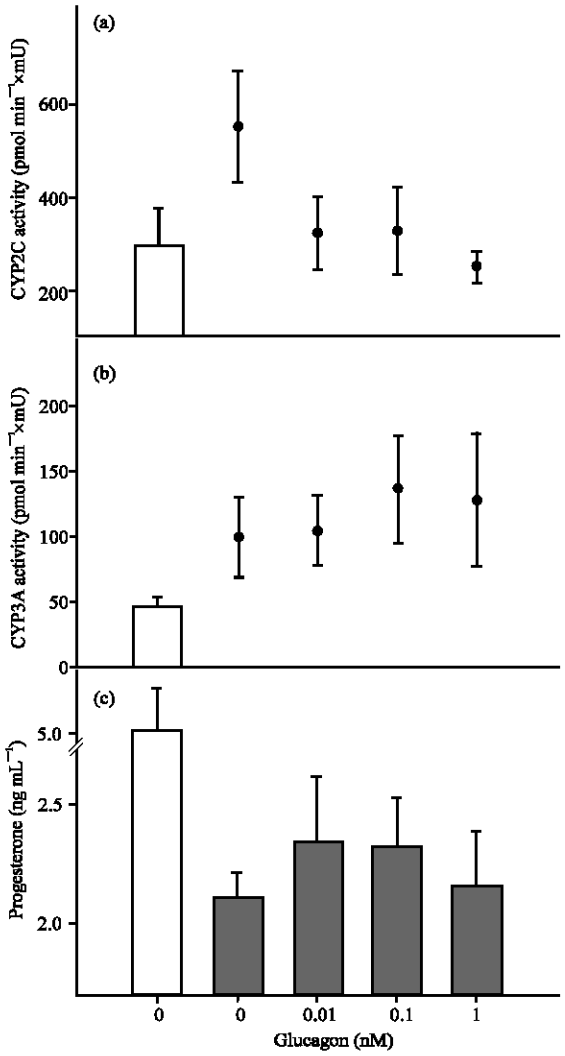

Fig. 2: Cytochrome P450 2C activity, 3A activity and progesterone concentrations in a mouse hepatocyte cell-line challenged with increasing concentrations of glucagon

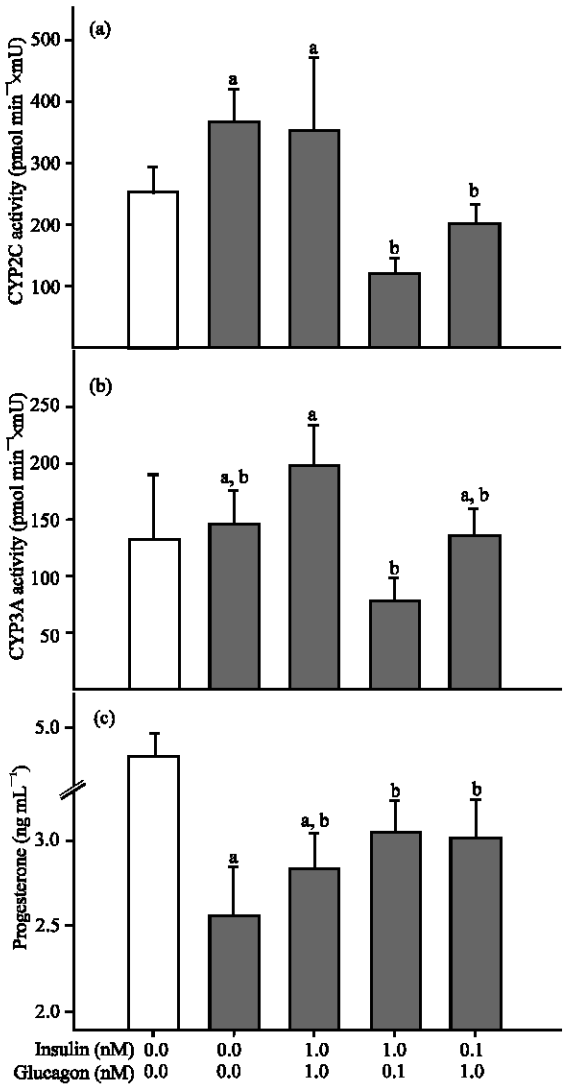

Fig. 3: Cytochrome P450 2C activity, 3A activity and progesterone concentrations in a mouse hepatocyte cell-line challenged with a combination of insulin and glucagon

hepatocytes cultured with 1 nM insulin had a trend ($p<0.1$) for elevated progesterone concentrations, while hepatocytes cultured with 0.1 and 10 nM insulin had elevated ($p<0.05$) progesterone concentrations compared to the 0 nM insulin treatment group (Fig. 1c).

Cytochrome P450 2C and 3A activities in mouse hepatocytes incubated for 4 h with varying concentrations of glucagon are illustrated in Fig. 2a and b, respectively. There was no observed effect of glucagon on CYP2C or CYP3A activity. Similarly progesterone concentrations in cell culture media after the 4 h challenge with glucagon were similar amongst treatment groups (Fig. 2c).

Cytochrome P450 2C and 3A activities in mouse hepatocytes incubated for 4 h in the presence of insulin and glucagon at different concentrations are illustrated in Fig. 3a 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 ($p<0.01$) compared to controls. Hepatocytes cultured with 0.1 nM insulin and 1.0 nM glucagon also showed a decrease ($p<0.05$) in CYP2C activity. Cytochrome P450 3A activity in hepatocytes cultured with 1.0 nM insulin and 0.1 nM glucagon tended

to belower ($p<0.1$) than the control groups (no insulin or glucagon) and was decreased ($p<0.01$) compared to CYP3A activity in hepatocytes cultured with 1.0 nM insulin and 1.0 nM glucagon. Progesterone concentrations in cell culture media after a 4 h incubation of hepatocytes with 5 ng mL^{-1} progesterone along with varying concentrations of insulin and glucagon are illustrated in Fig. 3c. Compared to the control group (0 nM insulin and 0 nM glucagon) cells challenged with 1 nM insulin and 1 nM glucagon had similar progesterone concentrations after the 4 h incubation. However, cells cultured with 1 nM insulin and 0.1 nM glucagon or 0.1 nM insulin and 1.0 nM glucagon had elevated ($p<0.05$) progesterone concentrations compared to the control group at the end of the 4 h incubation.

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 (Smith *et al*., 2006). Although, we did not measure the fractional rate constant of progesterone decay, as Smith *et al*. (2006), we did measure elevated progesterone concentrations after a 4 h incubation with 0.1 or 10 nM insulin compared to 0 nM insulin. 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 (Hewitt *et al*., 2007). Extensive studies exist on the enzymatic processes carried out by cytochrome P450s as well as the mechanisms behind their induction; however, only a limited number of studies have been focused on decreasing these enzymes without the use of toxic inhibitors (Hewitt *et al*., 2007).

It is well established that chemically induced or spontaneous insulin-dependent 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 (Barnnett *et al*., 1990). 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 (1999) showed that pyridine-mediated induction of CYP2E1 mRNA and protein abundance could be substantially decreased when cells were cultured in the presence of 1 μM insulin for 96 h; however, insulin exposure did not affect xenobiotic-mediated expression of CYP2B, CYP3A and CYP4A. By focusing on CYP3A1 expression rather than the entire CYP3A subfamily, Sidhu and Omiecinski (1999) found a decreased expression of CYP3A1 in rat hepatocytes cultured with 1 μM insulin and then exposed to phenobarbitol, a known CYP3A1 inducer in rats. They also found a lower induction of CYP3A1, due to phenobarbitol exposure, in cells exposed to physiological (e.g. 1 nM) concentrations of insulin (Sidhu and Omiecinski, 1999).

Saad *et al*. (1994) found a dose-dependent decrease in testosterone hydroxylation at the 6β 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 (Ryan and Levin, 1990). This is similar to our findings, which show a dose-dependent decrease in CYP2C and CYP3A activities using 0.1, 1 and 10 nM insulin concentrations. Formation of 6β hydroxytestosterone decreased 40% when cells were cultured in 10 nM insulin compared to 1 nM insulin (Saad *et al*., 1994), 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 and consequently progesterone concentrations at the end of the 4 h incubation were not different among treatment groups. 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. In contrast to our results hepatocytes exhibited a dose dependent decrease in 6β 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 6β 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). In contrast we found no differences in CYP2C or CYP3A activities after a glucagon challenge. Saad *et al*. (1994) found that exposing rat hepatocytes to insulin caused a greater decrease in hydroxylation of testosterone at the 6β 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 (1999) suggested that insulin and glucagon should be added to hepatocyte cultures at physiological concentrations when studying drug metabolism *in vitro*. Saad *et al.* (1994) showed a 20% decrease in 6β 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 (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. They found a greater response in CYP2B1 and CYP3A1 mRNA down-regulation 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 down-regulation 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 h (which is at least 20 h longer than our treatments). Liver exposure to elevated concentrations of insulin and glucagon may be as short as one or 2 h day^{-1}, which may be enough time to regulate activity, while having little to no effect on mRNA. 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 decreased enzymatic activity) at 30 and 60 min post-treatment at low dosages of cAMP, whereas treatment with higher concentrations of cAMP stimulated steroid catabolism, thereby suggesting both inhibitory and stimulatory roles for cAMP in steroid catabolism. Banhegyi *et al.* (1988) 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 (Miller and Yang, 1984; Koop and Casazza, 1985).

Regulation of CYP2C and CYP3A via insulin describes a logical system, which could facilitate lowered progesterone catabolism. You (2004) reviewed hepatic steroid hormone biotransformation as well as endocrine disruption due to cytochrome P450 induction. After the initial hydroxylation of progesterone by CYP2C or CYP3A the hydroxyprogesterone undergos a conjugation reaction carried out by the steroid-conjugating enzymes, which consist of sulfotransferases and the uridine diphosphate-glucuronosyltransferases (UGT) families (You, 2004). This added polarity aids in the excretion or elimination of the steroid from the body, typically in the form of pregnanediol.

CONCLUSION

Several Authors have shown that a decrease in liver blood flow, by feeding at half of maintenance requirements, increased 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 that allows for normal liver blood flow, while minimizing deleterious affects of starvation as a means to elevate 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. In conclusion, a logical mechanism to decrease progesterone catabolism, via cytochrome P450 down-regulation, in animals with excessive catabolism, could be used to increase concentrations of progesterone due to deficiencies in luteal secretion.

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