Hepatic glucocorticoid and α_1 - and β_2 -adrenergic receptors in calves change during neonatal maturation and are related to energy regulation

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ABSTRACT

Catecholamines and glucocorticoids are involved in fetal maturation of organ systems to prepare the fetus for extrauterine life. Calves, especially when born preterm, depend on function of the adrenergic system and the glucocorticoid axis to adapt energy metabolism for the neonatal period. We tested the hypothesis that hepatic glucocorticoid and α_1 - and β_2 -adrenergic receptors in neonatal calves are involved in adaptation of energy metabolism around birth and that respective binding capacities depend on stage of maturation during the neonatal period. Calves ($n = 7$ per group) were delivered by section preterm (PT, 9 d before term) or were born at term (full-term, FT; spontaneous vaginal delivery), or spontaneously born and fed colostrum for 4 d (FTC). Blood samples were taken immediately after birth and before and 2 h after feeding at 24 h after birth (PT, FT) or on d 4 of life (FTC) to determine metabolic and endocrine changes. After slaughter at 26 h after birth (PT, FT) or on d 4 of life (FTC), liver tissue was obtained to measure hepatic binding capacity of glucocorticoid and α_1 - and β_2 -adrenergic receptors. Maximal binding capacity and binding affinity were calculated by saturation binding assays using [³H]-prazosin and [³H]-CGP-12177 for determination of α_1 - and β_2 -adrenergic receptors, respectively, and [³H]-dexamethasone for determination of glucocorticoid receptor in liver. Additional liver samples were taken to measure mRNA abundance of glucocorticoid and α_1 - and β_2 -adrenergic receptors, of key enzymes and factors related to hepatic lipid metabolism, and of insulin-like growth factor 1 (*IGF1*). Plasma concentrations of β -hydroxybutyrate and leptin changed with time, and leptin concentrations were affected by stage of maturation. The binding capacities for hepatic glucocorticoid

and β_2 -adrenergic receptors as well as gene expression of *IGF1* were greater in FTC than in FT and PT, and binding affinity for β_2 -adrenergic receptor was lowest in PT. The binding capacity of hepatic α_1 -adrenergic receptor was greatest in FTC and greater in FT than in PT. The binding capacities of glucocorticoid and α_1 -adrenergic receptors were mainly related to variables of glucose and lipid metabolism. In conclusion, our results indicate dependence of hepatic glucocorticoid and adrenergic receptors on stage of maturation in neonatal calves and emphasize the association of α_1 -adrenergic receptor and glucocorticoid receptor with neonatal glucose and lipid metabolism.

Key words: calf, development, adrenergic receptor, glucocorticoid receptor, energy metabolism

INTRODUCTION

Neonatal growth and development depend on fetal maturation of vital organs, metabolic pathways, thermogenesis, and the immune system. By the end of gestation, glucocorticoids and catecholamines are strongly involved in these maturation processes, as indicated by increased adrenal weight relative to BW (Dalle et al., 1985) and by the increase in fetal glucocorticoids and catecholamines before birth (Girard et al., 1992; Liggins, 1994; Fowden and Forhead, 2011). In cattle, glucocorticoids and the adrenergic system are important regulators of energy metabolism (Brockman and Laarveld, 1986; Hammon et al., 2012) and both are responsible for maturation of processes and metabolic pathways involved in postnatal energy metabolism (Liggins, 1994; Fowden et al., 1998; Fowden and Forhead, 2011). Glucocorticoids (Pilkis and Granner, 1992; Edgerton et al., 2006) and the adrenergic system (Goodhardt et al., 1984; Apatu and Barnes, 1991; Fowden and Forhead, 2011) stimulate hepatic glucose production and may affect hepatic lipid metabolism (Rose et al., 2010). In calves, β -adrenergic agonists stimulated glycogenolysis and fat mobilization in a dose-dependent manner (Blum and Flueckiger, 1988), whereas treatment with dexamethasone

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methasone, a potent glucocorticoid, failed to stimulate postnatal endogenous glucose production, but provoked peripheral insulin resistance (Hammon et al., 2003).

The effects of glucocorticoids and the adrenergic system are mediated by specific receptors (Aggerbeck et al., 1980; Bylund et al., 1994; Edgerton et al., 2006). The dominant adrenergic receptor subtypes in the liver of neonatal calves are α_1 - and β_2 -adrenergic receptors, which are involved in postnatal maturation processes (Carron et al., 2005; Ontsouka et al., 2006), whereas hepatic glucocorticoid receptor has been investigated in neonatal calf liver and muscle with respect to colostrum feeding (Waggoner et al., 1988; Schäff et al., 2014).

Metabolic adaptation to extrauterine life is especially impaired in calves born preterm. These calves often suffer from hypoglycemia because endogenous glucose production is not fully developed (Girard, 1990; Bittrich et al., 2002; Steinhoff-Wagner et al., 2011), and responses to hormones such as cortisol and catecholamines are impaired around birth (Hammon et al., 2012, 2013). In calves and lambs born preterm, noradrenaline concentration especially was markedly increased in the first 10 d of life (Richet et al., 1985), thus preterm-born animals show a blunted metabolic response to plasma catecholamine concentrations (Padbury et al., 1987). Consequently, adaptation of energy metabolism to extrauterine life in preterm-born calves might be delayed due to reduced functioning of the glucocorticoid axis and the adrenergic system.

The aim of the present study was to clarify the associations of hepatic adrenergic and glucocorticoid receptor binding capacity and affinity with energy metabolism, especially endogenous glucose production, in neonatal calves with variable degrees of postnatal maturation. We investigated calves delivered by section 9 d before expected birth with reduced exposure to plasma cortisol during late fetal development (Steinhoff-Wagner et al., 2011), calves born at term with normal gestation length and physiological exposure to cortisol, and calves born at term and fed colostrum for 4 d. Binding studies of adrenergic and glucocorticoid receptors were related to parameters associated with glucose and lipid metabolism to determine the importance of individual receptors for energy metabolism in calves around birth.

MATERIALS AND METHODS

Animals and Feeding

The experimental procedures were carried out according to animal care guidelines and approved by the relevant authorities of the State Mecklenburg-Western Pomerania, Germany (LALLF M-V/TSD/7221.3–1.1–014/07).

In total, 21 German Holstein calves (20 male, 1 female) were studied and allocated to 3 different experimental groups ($n = 7$). The design of the study was described previously (Steinhoff-Wagner et al., 2011). Calves were spontaneously born (full-term; **FT**) or delivered by cesarean section 9 d before the expected day of calving (preterm; **PT**). Because of a previous investigation on basic rates of endogenous glucose production immediately after birth, calves of these 2 groups were bottle-fed once with colostrum 24 h after birth and slaughtered 26 h after birth (Steinhoff-Wagner et al., 2011). Calves of the third group were spontaneously born and bottle-fed colostrum (**FTC**) twice daily on d 1 and 2 of life and once daily at d 3 and 4 of life, and slaughtered 2 h after the morning feeding on d 4. Gestation length was 271.4 ± 0.5 , 283.7 ± 2.7 , and 281.3 ± 1.1 d for PT, FT, and FTC, respectively, and BW at birth was 41 ± 0.7 , 46.3 ± 2.5 , and 46.3 ± 1.3 kg for PT, FT, and FTC, respectively (Steinhoff-Wagner et al., 2011).

Amounts fed per meal in FTC were 4% of BW on d 1 and 5% of BW on d 2 to 4. The feeding 2 h before slaughter for all calves was at 5% of BW. Colostrum was collected before the trial started and individually pooled for milkings 1, 3, and 5 after calving (Steinhoff-Wagner et al., 2011), which was fed to FTC calves on d 1, 2, and 3, respectively. On d 4, FTC calves also received colostrum of milking 5 at 24 h after birth. Two hours after the final feeding, calves were slaughtered and liver tissue was collected and stored at -80°C until further analyses.

Metabolites and Hormones

Blood samples were taken by puncture or by catheter from the jugular vein right after birth, before last feeding (24 h after birth in PT and FT, 96 h after birth in FTC), and 2 h after feeding (Steinhoff-Wagner et al., 2011) in lithium heparin tubes to determine plasma cortisol and in K_3 EDTA tubes to measure concentrations of plasma glucose, NEFA, BHBA, leptin, IGF-I, 3,5,3'-triiodothyronine (**T₃**), and thyroxine (**T₄**). Samples were put on ice and then centrifuged ($1,500 \times g$, 4°C , 20 min), and plasma was stored at -20°C until analyzed. As described previously, plasma concentrations of glucose, NEFA, and BHBA were analyzed photometrically (Steinhoff-Wagner et al., 2011). Plasma insulin, IGF-I, **T₃**, and **T₄** concentrations were measured by RIA (Steinhoff-Wagner et al., 2011). Plasma leptin concentrations were determined by an enzyme immunoassay (Sauerwein et al., 2004). Plasma cortisol concentrations were analyzed using a commercially available ^{125}I -RIA kit (DSL Inc., Sinsheim, Germany) as described by Steinhoff-Wagner et al. (2011).

Glucose oxidation and endogenous glucose production were determined by intravenous [$U\text{-}^{13}\text{C}$]-glucose infusion immediately after birth (PT and FT) or on d 3 of life (FTC). Blood samples were taken over 4 h, and enrichment of [$U\text{-}^{13}\text{C}$]-glucose in plasma was measured by GC-MS (Steinhoff-Wagner et al., 2011). Endogenous glucose production was calculated as the rate of [$U\text{-}^{13}\text{C}$]-glucose appearance in calves fasted overnight. Enrichment of ^{13}C in blood CO_2 was measured as a proxy for glucose oxidation (Steinhoff-Wagner et al., 2011).

Analyses in Liver Tissue

Saturation Binding Assays for α_1 - and β_2 -Adrenergic Receptors. To prepare the membranes, approximately 20 g of crushed liver tissue was homogenized as described previously (Carron et al., 2005; Ontsouka et al., 2006) in 120 mL of ice-cold buffer (50 mM Tris-HCl buffer, pH 7.4, containing 6 mM magnesium chloride, and 1 mM EGTA) by using an Ultra-Turrax homogenizer (T25; IKA Werke, Staufen, Germany) on ice. Tissue suspensions were centrifuged at $800 \times g$ for 10 min at 4°C , the supernatant was centrifuged at $10,000 \times g$ for 10 min at 4°C , and thereafter the resulting supernatant was centrifuged at $30,000 \times g$ for 10 min at 4°C (Carron et al., 2005). The final pellet was dissolved in ice-cold buffer and homogenized with a tight-fitting Teflon-glass homogenizer (S 32 Homogenizator, Schütt Labortechnik, Göttingen, Germany) in an overhead stirrer (Eurostar digital, IKA Werke). The protein content of the tissue suspension was determined according to Bradford (1976), with BSA as standard. Samples were stored at -80°C until analysis.

Saturation binding assays were performed to determine the binding capacities of the antagonist [^3H]-prazosin for α_1 -adrenergic receptor (Bylund et al., 1994) and of the antagonist [^3H]-CGP-12177 for β_2 -adrenergic receptor (Bylund et al., 1994). Dilution series for adrenergic receptor saturation assay were prepared before each assay with the respective tracer [^3H]-prazosin (specific activity 85.3 Ci/mmol; Perkin Elmer, Waltham, MA; ranging from 0.05 to 2.9 nM) and [^3H]-CGP-12177 (specific activity 30 Ci/mmol; Perkin Elmer; ranging from 0.05 to 5.5 nM). To calculate nonspecific binding, phentolamine (Sigma Aldrich, St. Louis, MO) was used as specific competitor for α_1 -adrenergic receptor (Ontsouka et al., 2006), and the nonselective antagonist propranolol (Sigma Aldrich) was used as specific competitor for β_2 -adrenergic receptor (Blum and Flueckiger, 1988; Bylund et al., 1994; Carron et al., 2005). Competitors were diluted in buffer to a final concentration of 1 μM . Tissue suspension was adjusted to 2 mg of protein per mL with buffer. For total activity, tracer and buffer

were pipetted directly into scintillation vials. For total binding, tracer was added to buffer, and for nonspecific binding, tracer was added to competitor solution. After adding tissue, the suspension tubes were incubated for 2 h at 4°C under gentle shaking. Then, reactions were stopped by adding 1 mL of ice-cold buffer. For filtration, filter membranes (MNGF-3, i.d. 25 mm; Macherey-Nagel, Düren, Germany) were moistened with saline solution (9 g/L) and adjusted on a multiple filter device (H. Hölzel Labortechnik, Wörth, Germany). Prepared tissue solutions were added, tubes rinsed with saline solution, which was removed by vacuum pump. After washing each filter membrane 3 times with 5 mL of saline solution, almost-dry filters were covered with 3 mL of scintillator solution (Rotiszint eco plus, Carl Roth GmbH, Karlsruhe, Germany) in scintillation vials and measured on a Tri-Carb 2910TR Liquid Scintillation Analyzer (Perkin Elmer) at an efficiency of 50%. Each dilution was run in triplicate. For each adrenergic receptor, specific binding was calculated as the remaining amount of nonspecific binding minus the amount of total binding and was expressed as ratio of bound antagonist per membrane protein (fmol/mg). Equilibrium binding data were plotted as function of tracer concentration (saturation curve). Maximal binding capacity (B_{max}) and dissociation constant (K_d) were calculated by weighted least squares curve fitting using GraphPad Prism3 (GraphPad Software Inc., 1999). Scatchard plots (Scatchard, 1949) were calculated from specific [^3H]-prazosin binding for α_1 -adrenergic receptor and [^3H]-CGP-12177 binding for β_2 -adrenergic receptor to further define binding characteristics.

Saturation Binding Assays for Glucocorticoid Receptor. Glucocorticoid receptor binding in calf liver was determined as described previously by Kanitz et al. (2003) with a few modifications. Briefly, 1 g of crushed liver tissue powder was homogenized in ice-cold 10 mM Tris-HCl buffer, pH 7.5, containing 12.5 mM EDTA, 10 mM sodium molybdate, 0.25 mM saccharose, and 5 mM dithiothreitol using a Teflon-glass homogenizer. The homogenate was centrifuged at $120,000 \times g$ for 60 min at 0 to 4°C to obtain the cytosol (i.e., the supernatant fraction). The cytosol was treated with dextran-coated charcoal (1 g of charcoal and 0.1 g of dextran T70 dissolved in 10 mM Tris-HCl buffer, pH 7.5) for 30 min at 4°C to remove any excess of endogenous steroid. The supernatant was then centrifuged at $100,000 \times g$ for 10 min at 4°C and the resulting clear supernatant was used as the cytosol fraction for glucocorticoid receptor binding assays. Protein concentrations for each sample were determined by the method of Lowry et al. (1951).

Glucocorticoid receptor binding was evaluated directly in saturation experiments using the pure glucocorticoid [^3H]-dexamethasone (specific activity 43

Ci/mmol; Amersham Pharmacia Biotech, Freiburg, Germany) over a concentration range of 0.2 to 24 nM. Nonspecific binding was determined with a parallel incubation that contained a 2,000-fold excess of RU 28362 (kindly donated by Roussel Uclaf, Romainville, France), which binds selectively to the glucocorticoid receptor. The separation of bound from free ligand was performed by precipitation with dextran-coated charcoal, and the receptor-[³H]-steroid complexes were counted in a spectral liquid scintillation counter (Tri-Carb 2900TR, Perkin Elmer). The $B_{\max}$ and K_d for [³H]-dexamethasone binding in liver were derived from saturation experiments generated for each animal individually, and calculated by using MultiCalc (MultiCalc Software, Wallac, Finland) based on linear Scatchard analysis (Scatchard, 1949). In addition, saturation binding curves were created to compare saturation of binding between glucocorticoid receptor and adrenergic receptors.

Preparation of RNA and Real-Time Quantitative Reverse Transcription-PCR. Frozen liver tissue (50 mg) was pestled under liquid N₂ and homogenized with a FastPrep 120 centrifuge (Thermo Electron Corporation, Saint Herblain CEDEX, France). Tissue homogenate was used to extract total RNA using Trizol Reagent (Invitrogen, Carlsbad, CA). Integrity and quality of total RNA was confirmed upon gel electrophoresis on agarose gels stained with ethidium bromide and by measuring the optical density at 260:280, which was ≥ 1.9 with a spectrophotometer (NanoPhotometer; Implen, Munich, Germany). For cDNA synthesis, 1 μ g of RNA was reverse-transcribed with 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase, RNase H Minus, Point Mutant (Promega, Madison, WI) and 100 pmol of random primers (Invitrogen). The cDNA was purified with High Pure PCR Product Purification Kit (Roche Diagnostics, Laval, QC, Canada) and stored at -80°C . Specific primers were used to measure the mRNA abundance of α_1 -adrenergic receptor (**ADRA1**), β_2 -adrenergic receptor, (**ADRB2**), glucocorticoid receptor (**NR3C1**), acyl-CoA dehydrogenase very long chain (**ACADVL**), acyl-CoA synthetase long chain (**ACSL**), acyl-CoA dehydrogenase medium chain (**ACADM**), carnitine palmitoyltransferase 1 α (**CPT1A**), peroxisome proliferator-activated receptor α (**PPARA**), insulin-like growth factor 1 (**IGF1**), and phosphoglycerate kinase 1 (**PGK1**; Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2014-8636>), with **PGK1** serving as reference gene (because it was not affected by ontogenic state). For real-time quantitative reverse transcription (qRT)-PCR, 1 μ L of cDNA was used in a FastStart DNA Master Plus SYBR Green I MasterMix on a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). Each cDNA sample was ana-

lyzed in triplicate. To verify specific qRT-PCR products, a melting curve analysis program was performed after the last amplification cycle. Furthermore, product purity and size were confirmed by agarose gel electrophoresis showing a single band at the expected size, and by sequencing using an ABI Sequencing kit (ABI Big Dye Terminator, Applied Biosystems, Darmstadt, Germany) on an ABI 310 Genetic Analyzer (Applied Biosystems). Values of crossing point (quantification cycle; **Cq**) were corrected for different runs by an internal standard (ΔCq). In addition, $\Delta\text{Cq}_{\text{PGK1}}$ was used to normalize measurements of target genes as indicated by $\Delta\Delta\text{CP} = \Delta\text{Cq}_{\text{target}} - \Delta\text{Cq}_{\text{PGK1}}$ (Hammon et al., 2003). Efficiency of qRT-PCR was close to 2, and inter- and intraassay coefficients of variation of target and reference genes were $<1\%$ (Hammon et al., 2003; Steinhoff-Wagner et al., 2011).

Determination of Total Liver Fat and Glycogen Concentrations. Pestled liver tissue (50 mg) was dried for 3 h at 105°C , and DM content was determined. After analyzing nitrogen and carbon contents using an element CNS-2000 analyzer (Leco, Mönchengladbach, Germany), total liver fat concentration was calculated as previously described (Kuhla et al., 2004), whereas total liver glycogen concentrations were determined from wet tissue (25 mg) using an enzyme-based starch kit (no. 10207748035; Boehringer Mannheim, Mannheim, Germany).

Statistics

All results are presented as means or least squares means with pooled standard errors (SE). Data of measurements in liver tissue were evaluated with the general linear model procedure of SAS (SAS Institute Inc., 2009) with group as main effect. Data of blood metabolites and hormones were evaluated with the mixed model procedure of SAS, with group, time of blood sampling, and group $\times$ time interaction as fixed effects, and individual calf as random effect. Effects of feeding were calculated separately by using only samples on day of slaughter. Individual differences in both models were tested using the Tukey-Kramer adjustment.

Including all calves, the CORR procedure of SAS was used to calculate Pearson correlations between $B_{\max}$ and K_d values of glucocorticoid receptor, α_1 - and β_2 -adrenergic receptors, and hepatic fat and glycogen concentrations, as well as mRNA abundances of **ADRA1**, **ADRB2**, **NR3C1**, **ACADVL**, **ACADM**, **ACSL**, **CPT1A**, **PPARA**, **IGF1**, and gluconeogenic enzymes [pyruvate carboxylase (**PC**); phosphoenolpyruvate carboxykinase (**PEPCK**)-cytosolic isoform, **PCK1**; PEPCK-mitochondrial isoform, **PCK2**; glucose-6-phosphatase (**G6Pase**), **G6PC**]. Additionally, Pearson correlations

Table 1. Plasma concentrations of BHBA and leptin in calves born preterm (PT) or full-term (FT), or born full-term and fed colostrum (FTC)¹

Item	Time ²	Group			SE	P-value		
		PT	FT	FTC		Group	Time	Group × time
BHBA (mmol/L)	1	0.09	0.07	0.08	0.01	0.74 ³	0.02 ³	0.18 ³
	2	0.09	0.13	0.10				
	3	0.09	0.09	0.08		0.37 ⁴	0.10 ⁴	0.31 ⁴
Leptin (µg/L)	1	1.64	2.17	2.15	0.14	0.01 ³	0.001 ³	0.09 ³
	2	1.23 ^b	1.17 ^b	1.71 ^a				
	3	1.28 ^b	1.20 ^b	1.71 ^a		0.001 ⁴	0.47 ⁴	0.68 ⁴

^{a,b}LSM with different superscript letters differ between groups.

¹Values are LSM ± pooled SE; n = 7 per group.

²1 = after birth, 2 = before food intake on d 2 (PT, FT) or d 4 (FTC), 3 = 2 h after food intake on d 2 (PT, FT) or d 4 (FTC).

³ANOVA for measurements in blood plasma (times 1, 2, and 3).

⁴Periprandial: ANOVA for measurements on day of slaughter before and after feeding (times 2 and 3).

between receptor binding data and endogenous glucose production, glucose oxidation, enzyme activities of gluconeogenic enzymes (PC, PEPCK, and G6Pase) were calculated. All data evaluated in liver tissue collected at slaughter were correlated with data of blood metabolites and hormones from the last blood sampling before slaughter. Data for blood metabolites except leptin and BHBA, mRNA abundance and activity of gluconeogenic enzymes, endogenous glucose production, and glucose oxidation were recently published in a companion paper (Steinhoff-Wagner et al., 2011). In summary, PT calves had the lowest plasma concentrations of cortisol and T₃ at birth. Plasma glucose concentrations from d 1 to 2 decreased more, but plasma concentrations of lactate, urea, and the glucagon:insulin ratio were higher in PT than in FT and FTC calves. Endogenous glucose production and glucose oxidation, as well as hepatic glycogen concentrations and PEPCK activities, were lowest in PT calves, whereas hepatic PEPCK activities were greatest in FTC calves (Steinhoff-Wagner et al., 2011).

RESULTS

Plasma Concentrations of BHBA and Leptin

Plasma concentration of BHBA increased ($P < 0.05$) from birth until day of slaughter and tended to decrease ($P < 0.1$) after feed intake at day of slaughter but did not show group differences (Table 1). In general, plasma BHBA concentrations were close to the limit of detection in calves and, therefore, of low relevance for energy supply in neonatal calves. Plasma concentration of leptin decreased ($P < 0.05$) from birth to day of slaughter in all groups. On day of slaughter, leptin concentrations were higher ($P < 0.05$) in FTC than in PT and FT (Table 1).

Saturation Binding Assay

Specific binding of [³H]-prazosin and [³H]-CGP-12177 was a saturable process (Figure 1A, 1B) and Scatchard analyses of specific binding resulted in linear plots (Figure 2A, 2B). Saturation binding assays revealed increasing B_{max} of α₁-adrenergic receptor with maturation, indicated by the lowest B_{max} of α₁-adrenergic receptor ($P < 0.05$) in PT calves and the greatest B_{max} ($P < 0.05$) in FTC calves (Table 2). The B_{max} of β₂-adrenergic receptor was greatest ($P < 0.05$) in FTC calves, whereas binding affinity was lowest (greatest K_d value; $P < 0.05$) in PT calves (Table 2).

Specific binding of [³H]-dexamethasone was saturable (Figure 1C) and Scatchard analyses of specific binding resulted in linear plots (Figure 2C). Maximal binding capacity was greatest ($P < 0.05$) in FTC calves (Table 2).

Abundance of mRNA and Total Fat and Glycogen Concentrations in Liver

Abundance of *ADRA1*, *ADRB2*, and *NR3C1* mRNA did not differ among groups (Table 3). Concerning genes related to lipid metabolism, *ACADM* mRNA abundance indicated a trend for an ontogenic effect. Abundance of *IGF1* mRNA was higher ($P < 0.05$) in FTC than in PT and FT calves (Table 3).

Total liver fat concentration was higher in PT than in FT and FTC calves, but was not affected by feeding colostrum (3.94 ± 0.27 , 1.87 ± 1.03 , and $1.83 \pm 0.30\%$ of wet weight for PT, FT, and FTC, respectively; $P < 0.001$), whereas liver glycogen concentration was higher in FTC than in PT and FT calves (1.29 ± 0.26 , 2.03 ± 0.26 , and $4.16 \pm 0.28\%$ of wet weight for PT, FT, and FTC, respectively; $P < 0.001$; Steinhoff-Wagner et al., 2011).

Figure 1. Saturation binding assays of calves born preterm (PT, □) or full-term (FT, ■), or born full-term and fed colostrum (FTC, ●). Specific binding (SB) shown as mean $\pm$ SE for each group and concentration of (^3H) -prazosin (for α_1 -adrenergic receptor; A) and (^3H) -CGP-12177 (for β_2 -adrenergic receptor; B), and (^3H) -dexamethasone (for glucocorticoid receptor; C).

Correlations Between Specific Binding to α_1 - and β_2 -Adrenergic and Glucocorticoid Receptors, and Several Parameters Involved in Glucose and Lipid Metabolism

Correlations between $B_{\max}$ and K_d of adrenergic and glucocorticoid receptors with mRNA abundance of *ADRA1*, *IGF-I*, and *ACADM* as well as plasma leptin concentrations were calculated in this study (Figure 3). Further parameters involved in systemic and hepatic glucose and lipid metabolism (presented in Steinhoff-Wagner et al., 2011) were correlated with adrenergic and glucocorticoid receptors in liver. Only correlations with correlation coefficients >0.45 are shown. Maximal binding capacity of glucocorticoid receptor was positively correlated with $B_{\max}$ of α_1 -adrenergic receptor ($r = 0.57$, $P < 0.01$), $B_{\max}$ of β_2 -adrenergic receptor ($r = 0.50$, $P = 0.02$), plasma concentrations of leptin ($r = 0.50$, $P = 0.02$), and *ACADM* ($r = 0.53$, $P = 0.01$), and *IGF1* mRNA abundance ($r = 0.62$, $P < 0.01$) in liver. Maximal binding capacity of α_1 -adrenergic receptor was positively correlated with $B_{\max}$ of β_2 -adrenergic receptor ($r = 0.60$, $P < 0.01$), with mRNA abundance of *ADRA1* ($r = 0.46$, $P = 0.04$), *ACADM* ($r = 0.45$, $P = 0.04$), and *IGF1* ($r = 0.49$, $P = 0.02$) in liver, and with plasma concentrations of leptin ($r = 0.55$, $P < 0.01$). Whole-body endogenous glucose production and hepatic PEPCK enzyme activity were positively correlated with $B_{\max}$ of α_1 -adrenergic and glucocorticoid receptors and with K_d of β_2 -adrenergic receptor. In addition, several positive and negative correlations were found between α_1 -adrenergic and glucocorticoid receptor binding capacity with systemic and hepatic parameters of glucose and lipid metabolism (Figure 3).

DISCUSSION

During late gestation and the early neonatal period, calves undergo marked changes in their energy status, especially glucose status and endogenous glucose production (Steinhoff-Wagner et al., 2011). In our study, the importance of the glucocorticoid and adrenergic system in the liver of calves for neonatal maturation of energy metabolism was demonstrated by the increasing density of α_1 - and β_2 -adrenergic receptors, as well as glucocorticoid receptors, with increasing stage of maturation around birth. The greater number of adrenergic and glucocorticoid receptors in FTC than in FT calves was probably a combined effect of increasing maturation age and colostrum feeding. Recent findings in neonatal calves indicated that colostrum feeding per se influences binding capacities of α_1 -adrenergic and glucocorticoid receptors compared with feeding of milk with a comparable nutrient density but without

Figure 2. Scatchard plots created from binding studies of [3 H]-prazosin (for α_1 -adrenergic receptor; A), [3 H]-CGP-12177 (for β_2 -adrenergic receptor; B), and [3 H]-dexamethasone (for glucocorticoid receptor; C) in calves born preterm (PT, $\square$) or full-term (FT, $\blacksquare$), or born full-term and fed colostrum (FTC, $\bullet$). Individual plots represent means of 7 calves, respectively. SB = specific binding.

growth-promoting substances (Schäff et al., 2014). Binding affinity of β_2 -adrenergic receptors increases in calves born preterm to those born full term, which also indicates maturation processes of the hepatic adrenergic system. Gene expression of *ADRA1* was associated with α_1 -adrenergic receptor binding capacity, but gene expression of *ADRB2* and *NR3C1* was not related to β_2 -adrenergic and glucocorticoid receptor binding, respectively, indicating the importance of posttranscriptional regulation for β_2 -adrenergic and glucocorticoid receptors in liver.

Catecholamines in the liver act predominantly through α_1 -adrenergic receptors (Blair et al., 1979; Schmelck and Hanoune, 1980; Goodhardt et al., 1984) to modulate glucose production. The number of α_1 -adrenergic receptor binding sites was found to increase with age in rats during the early neonatal period (Lange et al., 1987; Huff et al., 1991) and in calves (Ontsouka et al., 2006), which supports our findings. Catecholamines, especially adrenaline, stimulate gluconeogenesis in cattle (Brockman and Laarveld, 1986). The strong relationship of hepatic α_1 -adrenergic receptor binding density with endogenous glucose production, liver glycogen concentration, hepatic PEPCK activity, and plasma glucose concentrations point to the involvement of α_1 -binding sites in the maturation of hepatic glucose metabolism of neonatal calves.

The number of β_2 -adrenergic receptor binding sites did not increase in the same manner as the number of α_1 -adrenergic receptor binding sites; that is, it did not change between calves born preterm and those born at full term, as reported previously (Carron et al., 2005). For hepatic β_2 -adrenergic receptor binding density, we found only a strong relationship with liver glycogen concentration. The missing difference of β_2 -adrenergic binding density in liver between PT and FT calves and the greater ratio of α_1 - to β_2 -adrenergic receptor in FT than in PT calves indicates a greater importance of α_1 - over β_2 -adrenergic receptor for mediating catecholamine effects in liver during the final gestational period, and illustrates the maturation of the hepatic adrenergic system around birth (McMillian et al., 1983). In addition, the inverse association of hepatic α_1 -adrenergic receptor with liver fat concentration and the concurrent positive association with *ACADM* mRNA may emphasize the importance of α_1 -adrenergic receptors on hepatic fatty acid oxidation in calves.

The abundance of glucocorticoid receptor is promoted by cortisol concentration (Gupta et al., 2003), which increases in calves around birth (Steinhoff-Wagner et al., 2011; Hammon et al., 2012). Although glucocorticoid receptor binding capacity correlated inversely with plasma cortisol, glucocorticoid receptor binding

Table 2. Maximal binding capacity ($B_{\max}$) and dissociation constant (K_d) of (^3H)-prazosin (for α_1 -adrenergic receptor), (^3H)-CGP-12177 (for β_2 -adrenergic receptor), and (^3H)-dexamethasone (for glucocorticoid receptor) derived from saturation binding assays with liver tissue of neonatal calves born preterm (PT) or full-term (FT), or born full-term and fed colostrum (FTC)¹

Item	Group			SE	P-value
	PT	FT	FTC		
$B_{\max}$ (fmol/mg of protein)					
α_1 -Adrenergic receptor	43.41 ^c	115.24 ^b	185.21 ^a	13.04	<0.001
β_2 -Adrenergic receptor	35.91 ^b	39.86 ^b	85.02 ^a	11.10	0.01
Glucocorticoid receptor	31.77 ^b	35.45 ^b	50.81 ^a	2.91	<0.001
K_d (nM)					
α_1 -Adrenergic receptor	0.09	0.14	0.15	0.03	0.30
β_2 -Adrenergic receptor	1.21 ^a	0.60 ^b	0.68 ^b	0.15	0.02
Glucocorticoid receptor	4.07	2.96	3.61	0.63	0.47

^{a-c}LSM with different superscript letters differ between groups.

¹Data shown are LSM and pooled SE.

density in liver was greatest in FTC calves, probably because of the longer exposure to cortisol in FTC calves (Steinhoff-Wagner et al., 2011). However, the number of glucocorticoid receptors did not differ between PT and FT calves, whereas FT calves had longer gestational age and greater exposure to plasma cortisol than PT calves. Therefore, the perinatal cortisol surge, but not the number of hepatic glucocorticoid receptors, is responsible for the maturational effects of the hepatic glucocorticoid system at the end of gestation. Recent findings in colostrum- and formula-fed neonatal calves illustrate the importance of colostrum intake for post-natal increase of hepatic glucocorticoid binding density (Schäff et al., 2014).

We found positive associations of glucocorticoid receptor binding capacity with endogenous glucose

production and hepatic PEPCK activity and glycogen storage, which showed the effect of glucocorticoid receptor numbers on the maturation of glucose metabolism in neonatal calves. Glucocorticoids increase the gluconeogenic capacity that begins during late fetal development (Fowden et al., 1993, 1998; Liggins, 1994). However, we detected negative associations of glucocorticoid receptor binding capacity with hepatic mRNA abundance of *PC* and *G6PC*, and a negative association of glucocorticoid receptor binding affinity with G6Pase activity. Decreased plasma NEFA concentrations and elevated *ACADM* mRNA abundance together with greater glucocorticoid receptor binding capacity in liver indicated involvement of glucocorticoid receptors in hepatic fatty acid oxidation. Direct effects of glucocorticoids on hepatic fatty acid oxidation are less

Table 3. Hepatic mRNA abundance of α_1 - and β_2 -adrenergic receptors and glucocorticoid receptor, as well as of enzymes involved in lipid metabolism of calves born preterm (PT) or full-term (FT), or born full-term and fed colostrum (FTC)¹

Item ²	Group			SE	P-value
	PT	FT	FTC		
<i>ADRA1</i>	0.17	0.33	0.33	0.06	0.11
<i>ADRB2</i>	0.11	0.11	0.08	0.03	0.76
<i>NR3C1</i>	1.08	0.82	1.04	0.13	0.32
<i>CPT1A</i>	0.78	1.16	0.98	0.15	0.25
<i>ACADVL</i>	1.02	0.87	1.19	0.22	0.60
<i>ACADM</i>	11.10	13.71	21.94	3.11	0.06
<i>ACSL</i>	8.93	10.11	11.38	1.64	0.58
<i>PPARA</i>	11.40	7.69	8.57	1.32	0.15
<i>IGF1</i>	0.11 ^b	0.14 ^b	0.34 ^a	0.05	0.01

^{a,b}LSM with different superscript letters differ between groups (general linear model procedure, Tukey test).

¹Data shown are LSM and pooled SE.

²*ADRA1* = α_1 -adrenergic receptor; *ADRB2* = β_2 -adrenergic receptor; *NR3C1* = glucocorticoid receptor; *CPT1A* = carnitine palmitoyltransferase 1 alpha; *ACADVL* = acyl-coenzyme A dehydrogenase very long chain; *ACADM* = acyl-coenzyme A dehydrogenase medium chain; *ACSL* = acyl-CoA synthetase long chain; *PPARA* = peroxisome proliferator activated receptor α ; *IGF1* = insulin-like growth factor 1.

Figure 3. Correlations between maximal binding sites ($B_{\max}$) and dissociation constant (K_d) of α_1 -adrenergic receptor, β_2 -adrenergic receptor, and glucocorticoid receptor and several parameters involved in glucose and lipid metabolism. Only correlations with $r > 0.45$ were shown. No correlations were found for K_d of α_1 -adrenergic receptor. Dashed lines indicate negative correlations, and solid lines indicate positive correlations. Given correlations are evaluated by SAS with $P < 0.05$. Data of underlined parameters are presented in this paper; data of parameters in gray were presented in our recent publication (Steinhoff-Wagner et al., 2011). T_3/T_4 = ratio of plasma concentration of 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4), T_4 = plasma concentration of thyroxine, IGF-I = plasma concentration of insulin-like growth factor 1, cortisol = plasma concentration of cortisol, leptin = plasma concentration of leptin, glucose = plasma concentration of glucose, NEFA = plasma concentration of non-esterified fatty acids, G6Pase enzyme activity = glucose-6-phosphatase enzyme activity in liver tissue, PEPCK enzyme activity = phosphoenolpyruvate carboxykinase enzyme activity in liver tissue, *G6PC* = hepatic mRNA abundance of glucose-6-phosphatase, *PC* = hepatic mRNA abundance of pyruvate carboxylase, *ACADM* = hepatic mRNA abundance of acyl-coenzyme A dehydrogenase medium chain, *IGF1* = hepatic mRNA abundance of IGF-1, *ADRA1* = hepatic mRNA abundance of α_1 -adrenergic receptor.

investigated in neonates, but glucocorticoid treatment in cultured hepatocytes from rabbit fetuses did not affect oleate oxidation (Girard et al., 1992).

Positive associations of glucocorticoid or adrenergic receptors with plasma concentrations of leptin, IGF-I, and the T_3/T_4 ratio indicated a positive relationship of glucocorticoid and adrenergic receptor binding sites with energy status of calves and with maturation of the endocrine system, which is involved in regulation of neonatal energy metabolism (Brockman and Laarveld, 1986; Hammon et al., 2012). Glucocorticoids are well known for their maturational effect on the somatotrophic axis during the neonatal period (Breier et al., 2000). A similar mechanism is conceivable for the adrenergic system in the liver (Fowden and Forhead, 2011). Thyroid hormones are involved in the maturation of neonatal glucose metabolism (Fowden et al., 2001) and, additionally, T_3 stimulates lipolysis (Malbon et al., 1978). Thyroid activity may thus affect α_1 -adrenergic receptor binding capacity (Metz et al., 1996) and is probably involved in the switch from β_2 - to α_1 -adrenergic receptor in liver during development (Rao et al., 1995).

Plasma leptin increases from preterm to full term and during the neonatal period in calves (Blum et al., 2005), and the expression of hepatic *IGF1* is promoted by leptin (Ingvarsen and Boisclair, 2001). Abundance of hepatic *IGF1* and plasma leptin concentrations increased with maturation, as FTC calves showed the greatest values for both. Greater IGF-I plasma concentrations in FTC calves (Steinhoff-Wagner et al., 2011) are probably the result of elevated hepatic IGF-I production, which is part of the postnatal maturation of the somatotrophic axis (Breier et al., 2000; Hammon et al., 2012).

In conclusion, hepatic α_1 -adrenergic receptors show a continuous increase from late gestation to early neonatal life, whereas β_2 -adrenergic and glucocorticoid receptors change predominantly during the early neonatal period, but not during late fetal development. Endogenous glucose production and hepatic gluconeogenic capacity are mainly associated with α_1 -adrenergic and glucocorticoid receptor binding density, indicating that these receptor binding sites contribute most to the effects of the hepatic adrenergic and glucocorticoid

system on neonatal maturation of energy metabolism in calves.

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