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Effects of colostrum versus formula feeding on hepatic glucocorticoid and α_1- and β_2-adrenergic receptors in neonatal calves and their effect on glucose and lipid metabolism

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ABSTRACT

Neonatal energy metabolism in calves has to adapt to extrauterine life and depends on colostrum feeding. The adrenergic and glucocorticoid systems are involved in postnatal maturation of pathways related to energy metabolism and calves show elevated plasma concentrations of cortisol and catecholamines during perinatal life. We tested the hypothesis that hepatic glucocorticoid receptors (GR) and α_1- and β_2-adrenergic receptors (AR) in neonatal calves are involved in adaptation of postnatal energy metabolism and that respective binding capacities depend on colostrum feeding. Calves were fed colostrum (CF; n = 7) or a milk-based formula (FF; n = 7) with similar nutrient content up to d 4 of life. Blood samples were taken daily before feeding and 2 h after feeding on d 4 of life to measure metabolites and hormones related to energy metabolism in blood plasma. Liver tissue was obtained 2 h after feeding on d 4 to measure hepatic fat content and binding capacity of AR and GR. Maximal binding capacity and binding affinity were calculated by saturation binding assays using [^{3}H]-prazosin and [^{3}H]-CGP-12177 for determination of α_1- and β_2-AR and [^{3}H]-dexamethasone for determination of GR in liver. Additional liver samples were taken to measure mRNA abundance of AR and GR, and of key enzymes related to hepatic glucose and lipid metabolism. Plasma concentrations of albumin, triacylglycerides, insulin-like growth factor I, leptin, and thyroid hormones changed until d 4 and all these variables except leptin and thyroid hormones responded to feed intake on d 4. Diet effects were determined for albumin, insulin-like growth factor I, leptin, and thyroid hormones. Binding capacity for GR was greater and for α_1-AR tended to be greater in CF than in FF calves. Binding affinities were in the same range for each receptor type. Gene expression of α_1-AR (*ADRA1*) tended to be lower in CF than FF calves. Binding capacity of GR was related to parameters of glucose and lipid metabolism, whereas β_2-AR binding capacity was negatively associated with glucose metabolism. In conclusion, our results indicate a dependence of GR and α_1-AR on milk feeding immediately after birth and point to an involvement of hepatic GR and AR in postnatal adaptation of glucose and lipid metabolism in calves.
Key words: calf, milk diet, adrenergic receptor, glucocorticoid receptor, energy metabolism

INTRODUCTION

After birth, changes in energy metabolism of neonates results in endogenous glucose production (**eGP**) and fat utilization to compensate for the loss of continuous glucose supply via the placenta (Girard et al., 1992). In neonatal calves, eGP, through gluconeogenesis and glycogenolysis, increases after birth to meet glucose demands, because lactose intake is not sufficient to meet postnatal glucose requirements (Girard, 1990; Steinhoff-Wagner et al., 2011b; Hammon et al., 2013). Due to fat intake by colostrum feeding, reflected by increasing plasma concentrations of triacylglycerides and several proteins related to lipid transport in colostrum-fed calves (Hammon and Blum, 1998; Rauprich et al., 2000; Herosimczyk et al., 2013), fat sources become an additional fuel to meet energy requirements in the neonate that requires adaptation of neonatal energy metabolism (Girard, 1990; Hammon et al., 2012).

Glucocorticoids and the adrenergic system are important regulators of energy metabolism (McDowell, 1983; Brockman and Laarveld, 1986) and are both responsible for maturation of processes and metabolic path-

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ways 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 eGP and may also affect FA oxidation (Olubadewo and Heimberg, 1993; Lettéron et al., 1997) and triacylglyceride storage in liver (Vegiopoulos and Herzig, 2007; 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, a potent glucocorticoid, failed to stimulate postnatal eGP, but provoked peripheral insulin resistance (Hammon et al., 2003; Scheuer et al., 2006).

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 (**AR**) subtypes in the liver of neonatal calves are α_1- and β_2-AR, which are involved in postnatal maturation of energy metabolism (Carron et al., 2005a,b; Ontsouka et al., 2006), whereas hepatic glucocorticoid receptors (**GR**) have scarcely been investigated in neonatal calves. However, postnatal energy status, specifically glucose and lipid metabolism, depends on diet, as indicated by an improved energy status in colostrum-fed calves and an impaired energy status in calves fed milk with comparable nutrient density as colostrum, but without bioactive growth-promoting factors (Blum, 2006; Hammon et al., 2012, 2013).

In view of the importance of the glucocorticoid and adrenergic system for the adaptation of glucose and lipid metabolism to postnatal life and dependency of postnatal energy metabolism on diet, we investigated the variation of hepatic AR and GR binding sites and gene expression in calves fed either colostrum or a milk-based formula with the same nutrient content, but almost no growth-promoting substances, such as hormones and growth factors, in 4-d old calves. We tested the hypothesis that hepatic α_1- and β_2-AR as well as hepatic GR are involved in adaptation of postnatal energy metabolism in neonatal calves and that receptor binding depends on diet. In addition to binding studies on hepatic AR and GR, liver samples were collected to investigate the gene expression of AR and GR and of key factors involved in hepatic energy metabolism. Plasma samples were taken for measurement of metabolites and hormones related to energy metabolism. Data on eGP and glucose oxidation as well as parameters with respect to systemic and hepatic glucose metabolism were previously published in a companion paper (Steinhoff-Wagner et al., 2011a).

MATERIALS AND METHODS

Animals and Feeding

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

For comparison of changes in energy metabolism related to different feeding regimens, 14 male German Holstein calves were studied and allocated randomly to 2 different experimental groups (n = 7). The design of the study was described previously (Steinhoff-Wagner et al., 2011a). Briefly, calves were spontaneously born and either fed colostrum (**CF**) or formula (**FF**) by bottle twice daily on d 1 and 2 of life and once daily on d 3 and 4 of life. Amounts fed per meal were 4% of BW on d 1 and 5% of BW on d 2 to 4. Cows from the barn were milked twice daily and colostrum was pooled for milkings 1, 3, and 5 after calving for d 1 (milking 1), d 2 (milking 3), and d 3 and 4 (milking 5). The milk-based formula (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany) had a macronutrient density similar to colostrum milkings, but almost without bioactive compounds. The composition of colostrum milkings and respective formulas is given in Supplemental Table S1 (http://dx.doi.org/10.3168/jds.2014-8359), as recently published in Steinhoff-Wagner et al. (2011a). To improve their immune status, all calves were fed egg-derived immunoglobulins (6 to 10 g/d; Globigen; EW Nutrition GmbH, Visbek, Germany). All calves were slaughtered at d 4 of life 2 h after feeding. Liver tissue was collected and stored at −80°C until further analysis.

Metabolites and Hormones

Blood samples were taken by puncture of the jugular vein at d 1 and by catheter on d 2 to 4 right before and 2 h after feeding on d 4 in lithium heparin-containing tubes for determination of plasma cortisol concentration and in K_3 EDTA-containing tubes for measurement of plasma concentrations of glucose, total protein, albumin, urea, NEFA, triacylglycerides, BHBA, leptin, IGF-I, 3,5,3′-triiodothyronine ($\mathbf{T_3}$), and thyroxine ($\mathbf{T_4}$). Samples were put on ice, centrifuged (1,500 × g for 20 min at 4°C), and plasma was stored at −20°C until analyzed. As described previously, plasma glucose, total protein, albumin, urea, NEFA, triacylglycerides, and BHBA were analyzed photometrically (Steinhoff-Wagner et al., 2011b); plasma insulin, IGF-I, T_3, and T_4 concentrations were measured by RIA (Vicari et al.,

2008); and 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. (2011b).

Glucose oxidation and eGP were determined by intravenous [U-^{13}C]-glucose infusion on d 3 of life. Blood samples were taken during 8 h and enrichment of [U-^{13}C]-glucose in plasma was measured by GC-MS (Steinhoff-Wagner et al., 2011a). Endogenous glucose production was calculated as rate of [U-^{13}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., 2011a).

Analyses in Liver Tissue

Saturation Binding Assays for α_1-AR and β_2-AR. To prepare the membranes, approximately 20 g of crushed liver tissue was homogenized with a Teflon-glass homogenizer as described earlier (Carron et al., 2005a,b; Ontsouka et al., 2006) in 120 mL of ice-cold buffer, consisting of 50 m*M* Tris-HCl buffer (pH 7.4), containing 6 m*M* magnesium chloride and 1 m*M* ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), using an Ultra-Turrax homogenizer (T25; IKA Werke GmbH & Co. KG, Staufen, Germany) on ice. Tissue suspensions were centrifuged at 800 × *g* for 10 min at 4°C, the supernatant was centrifuged at 10,000 × *g* for 10 min at 4°C, and thereafter the resulting supernatant was again centrifuged at 30,000 × *g* for 90 min at 4°C (Carron et al., 2005a). The final pellet was dissolved in ice-cold buffer and homogenized with a tight-fitting Teflon-glass homogenizer (S 32 homogenizer; Schütt Labortechnik GmbH, Göttingen, Germany) in an overhead stirrer (Eurostar digital; IKA Werke GmbH & Co. KG). The protein content of the tissue suspension was determined according to Bradford (1976), with BSA as the standard. Samples were stored at −80°C until analysis.

Saturation binding assays were performed to determine the binding capacities of [^{3}H]-prazosin for α_1-AR (Bylund et al., 1994) and of [^{3}H]-CGP-12177 for β_2-AR (Bylund et al., 1994). Dilution series for AR saturation assay were prepared before each assay with the respective tracer [^{3}H]-prazosin [specific activity = 3,156.1 GBq/mmol; PerkinElmer LAS (Germany) GmbH, Rodgau, Germany; ranging from 0.05 to 2.9 n*M*] and [^{3}H]-CGP-12177 [specific activity = 1,110 GBq/mmol; PerkinElmer LAS (Germany) GmbH; ranging from 0.05 to 5.5 n*M*]. For calculations, phentolamine was used as a competitor for α_1-AR (Ontsouka et al., 2006) and propranolol for β_2-AR (Blum and Flueckiger, 1988; Bylund et al., 1994; Carron et al., 2005a). Competitors were diluted in buffer to a final concentration of 1 μ*M*. The tissue solution was set on 2 mg of protein/mL with buffer. For total activity, the tracer and buffer were pipetted directly into scintillation vials. For total binding, the tracer was added to the buffer, and for nonspecific binding, the tracer was added to the competitor solution. After adding tissue, the solution 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, 25-mm i.d.; Macherey-Nagel GmbH & Co. KG, Düren, Germany) were moistened with saline solution and adjusted on a multiple filter device (H. Hölzel Laborgeräte GmbH, Wörth, Germany). Prepared tissue solutions were added and tubes were rinsed with saline solution and sucked off. 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 & Co. KG, Karlsruhe, Germany) in scintillation vials and measured on a Tri-Carb 2910TR Liquid Scintillation Analyzer [PerkinElmer LAS (Germany) GmbH]. Each dilution was run in triplicate. For each AR, specific binding was calculated as the remaining amount of nonspecific binding minus the amount of total binding and was expressed as the ratio of bound antagonist per membrane protein (fmol/mg). Equilibrium binding data were plotted as a function of tracer concentration (saturation curve). Maximal binding capacity ($\mathbf{B_{max}}$) and dissociation constant ($\mathbf{K_d}$) were calculated by weighted least squares curve fitting using GraphPad Prism3 software (GraphPad Software Inc., 1999). Scatchard plots (Scatchard, 1949) were calculated from specific [^{3}H]-prazosin binding for α_1-AR and [^{3}H]-CGP-12177 binding for β_2-AR to further define binding characteristics.

Saturation Binding Assays for Glucocorticoid Receptors. Glucocorticoid receptor binding in calf liver was performed as previously described by Kanitz et al. (2003), with few modifications. Briefly, 1 g of crushed liver tissue powder was homogenized in ice-cold 10 m*M* Tris-HCl buffer (pH 7.5), containing 12.5 m*M* EDTA, 10 m*M* sodium molybdate, 0.25 m*M* sucrose, and 5 m*M* dithiothreitol, using a Teflon-glass homogenizer. The homogenate was centrifuged at 120,000 × *g* for 60 min at 0 to 4°C to obtain cytosol (i.e., the supernatant fraction). The cytosol was treated with dextran-coated charcoal (1 g of charcoal and 0.1 g of dextran T 70 dissolved in 10 m*M* Tris-HCl buffer, pH 7.5) for 30 min at 4°C to remove any excess endogenous steroid. The supernatant was then centrifuged at 100,000 × *g* for 10 min at 4°C and the resulting clear supernatant was

used as the cytosol fraction for GR binding assays. Protein concentrations for each sample were determined according to Lowry et al. (1951).

Glucocorticoid receptor binding was evaluated directly in saturation experiments using the pure glucocorticoid [^{3}H]-dexamethasone (specific activity = 1,591 GBq/mmol; Amersham Pharmacia Biotech AB, Freiburg, Germany) over a concentration range of 0.2 to 24 n*M*. Nonspecific binding was determined with a parallel incubation that contained 2,000-fold excess RU 28362 (kindly donated by Roussel Uclaf SA, Romainville, France), which binds selectively to the GR. The separation of bound from free ligand was performed by precipitation with dextran-coated charcoal and the receptor-[^{3}H]-steroid complexes were counted in a spectral liquid scintillation counter [Tri-Carb 2900TR; PerkinElmer LAS (Germany) GmbH] at an efficiency of 50%. The B_{max} and K_d for [3H]-dexamethasone binding in liver were derived from saturation experiments generated for each calf liver individually, and calculated using MultiCalc software (Wallac Oy, Turku, Finland) based on linear Scatchard analysis (Scatchard, 1949). In addition, saturation binding curves were created to compare saturation of binding between GR and AR.

Preparation of RNA and Real-Time Quantitative Reverse-Transcription PCR. Powdered liver tissue (50 mg) was homogenized with a FastPrep 120 centrifuge (Thermo Electron Corp., Saint-Herblain Cedex, France) and used to extract total RNA with TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Integrity and quality of total RNA was confirmed upon gel electrophoresis on agarose gel stained with ethidium bromide and by measuring the optical density ratio of absorbance at 260 and 280 nm (260:280), which was <1.9, with a spectrophotometer (NanoPhotometer; Implen GmbH, Munich, Germany). For cDNA synthesis, 1 μg of RNA was reverse transcribed with 200 U of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega Corp., Madison, WI) and 100 pmol of Random Primers (Invitrogen Corp.). The cDNA was purified using a 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-AR (***ADRA1***), β_2-AR (***ADRB2***), GR (***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***), and phosphoglycerate kinase 1 (***PGK1***; Table 1), with *PGK1* serving as reference gene, as it was not affected by diet. For quantitative reverse-transcription PCR, 1 μL of cDNA was used in a FastStart DNA Master Plus SYBR Green I MasterMix on a LightCycler 2.0 (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Each cDNA sample was analyzed in triplicate. To verify specific 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, ΔCq_{PGK1} was used to normalize measurements of target genes as indicated by $\Delta\Delta CP = \Delta Cq_{target} - \Delta Cq_{PGK1}$ (Hammon et al., 2003). Efficiency of quantitative reverse-transcription 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., 2011b).

Determination of Liver Glycogen Concentration and Liver Fat Concentration. Frozen liver tissue (50 mg) was mortared under liquid N_2, and DM was determined after drying for 3 h at 105°C. After analyzing nitrogen and carbon content with an element CNS-2000 analyzer (Leco Instrumente GmbH, Mönchengladbach, Germany), liver fat concentration (**LFC**) was calculated as previously described (Kuhla et al., 2004), whereas liver glycogen concentration (**LGC**) was determined from wet tissue (25 mg) using an enzyme-based starch kit (no. 10207748035; Boehringer Mannheim GmbH, Mannheim, Germany).

Enzyme Activity of Gluconeogenic Enzymes. Activities of phosphoenolpyruvate carboxykinase (**PEPCK**; EC 4.1.1.32) were determined in cell homogenates where carboxylation of phosphoenolpyruvate to oxaloacetate was measured using $NaH^{14}CO_3$ (Ballard and Hanson, 1967). The addition of NADH and malate dehydrogenase ensured the conversion of the reaction product, oxaloacetate, to malate (Atkin et al., 1979). Activity of pyruvate carboxylase (**PC**; EC 6.4.1.1) was assayed in the crude homogenate by $NaH^{14}CO_3$ incorporation into oxaloacetate and citrate in the presence of pyruvate (Atkin et al., 1979). Activities of glucose-6-phosphatase (**G6Pase**; EC 3.1.3.9) were measured according to Gierow and Jergil (1980). Liver samples were homogenized in 20 m*M* HEPES buffer containing 100 m*M* sucrose and 0.25 m*M* EDTA (wt/vol = 1:10) and glucose-6-phosphate served as substrate. Produced phosphate concentrations were determined by the reaction with ammonium molybdate and compared with a phosphate standard curve (Hammon et al., 2003). Data

Table 1. Primer sequences used for the relative quantification of target cDNA

Gene[1]	Primer sequence 5′–3′ [2]	Tm[3] (°C)	Amplicon size (bp)	Annealing conditions (°C/s)	MeltingTm[4] (°C)	Source	GenBank accession no.
PGK1	F: CAGTGGAGCCAAGTCAGTTG	63.5	87	60/10	85.6 ± 0.0	Steinhoff-Wagner et al. (2011a)	NM_001034299.1
	R: GCAACTGGCTGCAAGGAGTA	65.4	87	60/10	85.6 ± 0.0		
ADRA1	F: AAGAAAGCGGCCAAAACG	65.0	183	60/20	86.3 ± 0.1	Ontsouka et al. (2006)	J05426.1
	R: GCATGGGTATATAATGGGGTTG	63.5	183	60/20	86.3 ± 0.1		
ADRB2	F: TCATGTCGCTTATTGTCCTG	64.8	202	60/20	88.3 ± 0.1	Inderwies et al. (2003)	Z86037
	R: CACCAGAAGTTGCCAAAAGTCC	66.7	202	60/20	88.3 ± 0.1		
NR3C1	F: GTTTCTGCGTCTTCACCCTCA	61.0	160	56/20	85.6 ± 0.1	Chen et al. (2006)	BTU37383
	R: GTCTCTCCCATATACAGTCCC	61.0	160	56/20	85.6 ± 0.1		
ACADVL	F: TCCCCAAACTGGCATCTGGG	63.0	275	60/30	88.0 ± 0.0	van Dorland et al. (2009)	BC103104
	R: ATGGGTGACGCCGCCAAAGC	65.0	275	60/30	88.0 ± 0.0		
ACADM	F: GTGGAGGTCTTGGACTTG	56.0	176	51/20	79.7 ± 0.0	Graber et al. (2010)	NM_001075235.1
	R: ATGGCTCCTCAGTCATTCTC	58.0	176	51/20	79.7 ± 0.0		
ACSL	F: GGAGGTGCCGGGCAGTGATG	67.0	204	60/30	86.6 ± 0.0	van Dorland et al. (2009)	BC119914
	R: GCCATATCTTCAACCTGTTTG	57.0	204	60/30	86.6 ± 0.0		
CPT1A	F: CAAAACCATGTTGTACAGCTTCCA	62.0	111	54/30	87.9 ± 0.1	van Dorland et al. (2009)	BF039285
	R: GCTTCCTTCATCAGAGGCTTCA	62.0	111	54/30	87.9 ± 0.1		
PPARA	F: AGGGCTGCAAGGGTTTCTTTAG	62.0	363	60/30	84.6 ± 0.0	Graber et al. (2010)	NM_001034036
	R: TGACGAAAGGCGGGTTGTTGTTG	65.0	363	60/30	84.6 ± 0.0		
IGF1	F: TCGCATCTCTTCTATCTGGCCCTGT	64.6	240	62/10	90.3 ± 0.0	Pfaffl et al. (2002)	NM_001077828.1
	R: GCAGTACATCTCCAGCCTCCTCAGA	66.3	240	62/10	90.3 ± 0.0		

[1]*PGK1* = phosphoglycerate kinase 1; *ADRA1* = α_1-adrenergic receptor; *ADRB2* = β_2-adrenergic receptor; *NR3C1* = glucocorticoid receptor; *ACADVL* = acyl-CoA dehydrogenase very long chain; *ACADM* = acyl-CoA dehydrogenase medium chain; *ACSL* = acyl-CoA synthetase long chain; *CPT1A* = carnitine palmitoyltransferase 1α; *PPARA* = peroxisome proliferator-activated receptor α.

[2]F = forward; R = reverse.

[3]Melting temperature of the primer.

[4]Melting temperature, determined using LightCycler (Roche Diagnostics Deutschland GmbH, Mannheim, Germany).

for enzyme activities of PEPCK, PC, and G6Pase were recently presented in a companion paper (Steinhoff-Wagner et al., 2011a).

Statistics

All results are presented as least squares means with pooled standard error of the mean. Data of measurements in liver tissue were analyzed with PROC GLM of SAS (SAS Institute, 2009), with group as main effect. Data of blood metabolites and hormones were analyzed with PROC MIXED of SAS, with group, time of blood sampling, and group × time interaction as fixed effects, and individual calf as random effect. Differences among metabolites and hormones in basal blood samples (at 0, 24, 48, and 72 h after birth and before feed intake, respectively) and differences among metabolites and hormones before and after feed intake on d 4 (72 and 74 h after birth) were calculated separately. Individual differences in both models were tested using the Tukey-Kramer procedure.

The CORR procedure of SAS was used to calculate Pearson correlations between B_{max} and K_d values of GR, α_1-AR, β_2-AR, LGC, and LFC, and mRNA abundance of *ADRA1*, *ADRB2*, *NR3C1*, *ACADVL*, *ACADM*, *ACSL*, *CPT1A*, *PPARA*, and *IGF1* as well as mRNA abundance of gluconeogenic enzymes [*PC*; phosphoenolpyruvate carboxykinase, cytosolic isoform (***PCK1***); phosphoenolpyruvate carboxykinase, mitochondrial isoform (***PCK2***); and glucose-6-phosphatase (***G6PC***)]. Additionally, Pearson correlations between receptor binding data and eGP, glucose oxidation, and enzyme activities of gluconeogenic enzymes (PC, PEPCK, and G6Pase) were calculated. All data evaluated in liver tissue gained at slaughter were correlated to data of blood metabolites from the last sampling before slaughter (74 h after birth). All correlations were performed independent of diet and data for mRNA abundance and activities of gluconeogenic enzymes, eGP, and glucose oxidation as well as plasma concentrations of glucose, NEFA, insulin, glucagon, and cortisol were recently published in a companion paper (Steinhoff-Wagner et al., 2011a).

RESULTS

Gestation length was 281.1 ± 0.7 d, BW on d 1 was 45.9 ± 1.0 kg, and BW before slaughtering on d 4 was 46.8 ± 1.2 kg. Gestation length, BW, and health status of calves were not different between groups. Data were recently published in a companion paper (Steinhoff-Wagner et al., 2011a).

Plasma Hormones and Metabolites

Plasma concentrations of albumin decreased ($P < 0.001$) with time and after feeding ($P < 0.05$) on d 4, but were greater ($P < 0.05$) in FF than in CF calves (Table 2). Plasma triacylglyceride concentrations increased with time ($P < 0.01$) and increased ($P < 0.001$) after feed intake on d 4 only in FF calves (Table 2). Plasma concentrations of BHBA decreased (interaction on d 4; $P < 0.05$) after feed intake on d 4 only in CF calves (Table 2). Plasma concentrations of IGF-I changed with time during the first days of life ($P < 0.001$) and after feeding on d 4 ($P < 0.05$), but were lower ($P < 0.05$) on d 4 in FF than in CF calves (Table 2). Plasma concentrations of leptin decreased with time ($P < 0.01$) and were lower ($P < 0.01$) during the first days of life and on d 4 in FF than in CF calves (Table 2). Plasma concentrations of T_3 and T_4 decreased with time and were higher ($P < 0.05$) in CF than in FF calves on d 4 (Table 2).

Saturation Binding Assay

Specific binding of [^{3}H]-prazosin and [^{3}H]-CGP-12177 was a saturable process and Scatchard analyses of specific binding resulted in linear plots (Figure 1A and B; Figure 2A and B). The maximal binding capacity of α_1-AR in the liver tended to be greater ($P < 0.1$) in CF than in FF calves, but diets did not affect β_2-AR (Table 3). In addition, K_d values did not differ between groups for α_1- and β_2-AR (Table 3).

Specific binding of [^{3}H]-dexamethasone was saturable and Scatchard analyses of specific binding resulted in linear plots (Figure 1C; Figure 2C). Maximal binding capacity of GR was greater ($P < 0.05$) in CF than in FF calves, but K_d of GR between groups were in the same range (Table 3).

Measurement of mRNA Abundance in the Liver

Abundance of *ADRA1* mRNA tended to be lower ($P < 0.1$) in CF than in FF calves, whereas mRNA abundance of *ADRB2* and *NR3C1* as well as enzymes involved in lipid metabolism, *PPARA*, and *IGF1* were not affected by diet (Table 4).

Liver Fat and Glycogen Concentrations

Liver fat concentrations were comparable between CF and FF calves (18.3 vs. 18.1 ± 0.28 mg/g, wet weight), whereas LGC was greater in CF than in FF calves (399 vs. 194 ± 26 mg/g, wet weight; $P < 0.001$).

Table 2. Plasma metabolites and hormones of calves fed either colostrum (CF) or formula (FF)[1]

					P-value[3]					
		Diet			Group		Time		Group × time	
Item	Time[2]	CF	FF	SEM	d 1–4	d4; periprandial	d 1–4	d4; periprandial	d 1–4	d4; periprandial
Albumin (g/L)	0	24.81	26.10	0.36	<0.001	0.19	<0.001	0.02	<0.001	0.23
	24	22.34	26.20							
	48	22.50	25.06							
	72	23.44	23.90							
	74	22.66	23.36							
Triacylglycerides (mmol/L)	0	0.25	0.27	0.05	0.90	0.12	<0.01	<0.01	0.14	<0.001
	24	0.25	0.32							
	48	0.40	0.42							
	72	0.46	0.34							
	74	0.36	0.83							
BHBA (mmol/L)	0	0.08	0.09	0.01	0.55	0.79	0.19	0.18	0.28	0.04
	24	0.08	0.09							
	48	0.09	0.10							
	72	0.10	0.09							
	74	0.08	0.09							
IGF-I (μg/L)	0	155.5	147.5	14.2	0.41	0.02	<0.001	0.05	<0.01	0.08
	24	180.7	204.3							
	48	138.7	104.2							
	72	93.8	54.8							
	74	84.4	54.1							
Leptin (μg/L)	0	2.15	1.79	0.18	<0.01	<0.001	<0.01	0.32	0.13	0.50
	24	2.51	1.53							
	48	2.18	1.36							
	72	1.72	1.10							
	74	1.70	1.04							
3,5,3′-Triiodothyronine (nmol/L)	0	12.33	11.57	1.15	0.06	0.02	<0.001	0.46	0.38	0.81
	24	9.67	6.87							
	48	8.60	4.68							
	72	5.16	2.81							
	74	5.29	2.88							
Thyroxine (nmol/L)	0	289.6	281.2	25.0	0.19	<0.01	<0.001	0.38	0.47	0.46
	24	242.1	202.8							
	48	192.2	129.7							
	72	138.1	80.2							
	74	133.5	79.7							

[1]Data were evaluated by PROC MIXED of SAS (SAS Institute, 2009) and are presented as LSM and pooled SEM.

[2]Hours after birth.

[3]d 1–4: ANOVA for daily measurements (0, 24, 48, and 72 h after birth). d4; periprandial: ANOVA for measurements on day of slaughter before and after feeding (72 and 74 h after birth).

Correlations Between Specific Binding to α_1-AR, β_2-AR, and GR, and Several Parameters Involved in Glucose and Lipid Metabolism

Maximal binding capacity of β_2-AR was negatively correlated with eGP (r = −0.68; $P < 0.01$) and glucose oxidation (r = −0.80; $P < 0.001$). Maximal binding capacity of GR was positively correlated with PEPCK activity in the liver (r = 0.61; $P = 0.02$), hepatic *ACADM* (r = 0.57; $P = 0.04$), and plasma concentrations of glucose (r = 0.60; $P = 0.02$), insulin (r = 0.57; $P = 0.04$), and IGF-I (r = 0.55; $P = 0.04$), but was negatively correlated with plasma concentrations of NEFA (r = −0.55; $P = 0.04$).

Abundance of *ADRA1* mRNA was positively correlated with mRNA abundance of *IGF1* (r = 0.55; $P = 0.04$), *PCK2* (r = 0.82; $P < 0.001$), *CPT1A* (r = 0.68; $P < 0.01$), and *PPARA* (r = 0.59; $P = 0.03$), and with plasma urea concentrations (r = 0.61; $P = 0.02$), but was negatively correlated with plasma leptin concentration (r = −0.60; $P = 0.02$) and with LGC (r = −0.59; $P = 0.03$). Hepatic *NR3C1* abundance was positively correlated with mRNA abundance of *IGF1* (r = 0.80; $P < 0.01$) and *PPARA* (r = 0.67; $P < 0.01$) and with plasma concentrations of urea (r = 0.60; $P = 0.02$), glucagon (r = 0.86; $P < 0.001$), and cortisol (r = 0.76; $P < 0.01$).

Hepatic mRNA abundance of *CPT1A* and *PPARA* were in addition positively correlated with mRNA abundance of *PC* (*CPT1A*: r = 0.54 and $P = 0.05$; *PPARA*: r = 0.53 and $P = 0.05$), *PCK2* (*CPT1A*: r = 0.68 and $P < 0.01$; *PPARA*: r = 0.61 and $P = 0.02$), *G6PC* (*CPT1A*: r = 0.69 and $P < 0.01$; *PPARA*: r = 0.54 and $P = 0.05$), *IGF1* (*CPT1A*: r = 0.71 and $P < 0.01$; *PPARA*: r = 0.81 and $P < 0.001$), and *ACADM* (*CPT1A*: r = 0.72 and $P < 0.01$; *PPARA*: r = 0.80 and $P = 0.001$), and with plasma concentrations of BHBA (*CPT1A*: r = 0.56 and $P = 0.04$; *PPARA*: r = 0.56 and $P = 0.04$). Abundance of hepatic *ACADM* mRNA was positively correlated with *PCK2* mRNA (r = 0.73; $P < 0.01$) and *IGF1* mRNA (r = 0.66; $P = 0.01$), and with plasma IGF-I concentrations (r = 0.60; $P = 0.02$).

Liver glycogen concentration was positively correlated with plasma concentrations of leptin (r = 0.82; $P < 0.001$), IGF-I (r = 0.57; $P = 0.04$), T_3 (r = 0.55; $P = 0.04$), T_4 (r = 0.54; $P = 0.05$), and insulin (r = 0.58; $P = 0.03$), and was negatively correlated with plasma cortisol (r = −0.57; $P = 0.04$).

DISCUSSION

Postnatal maturation and growth in calves largely depends on colostrum feeding (Blum, 2006; Hammon et al., 2012, 2013). Besides insufficient immune status,

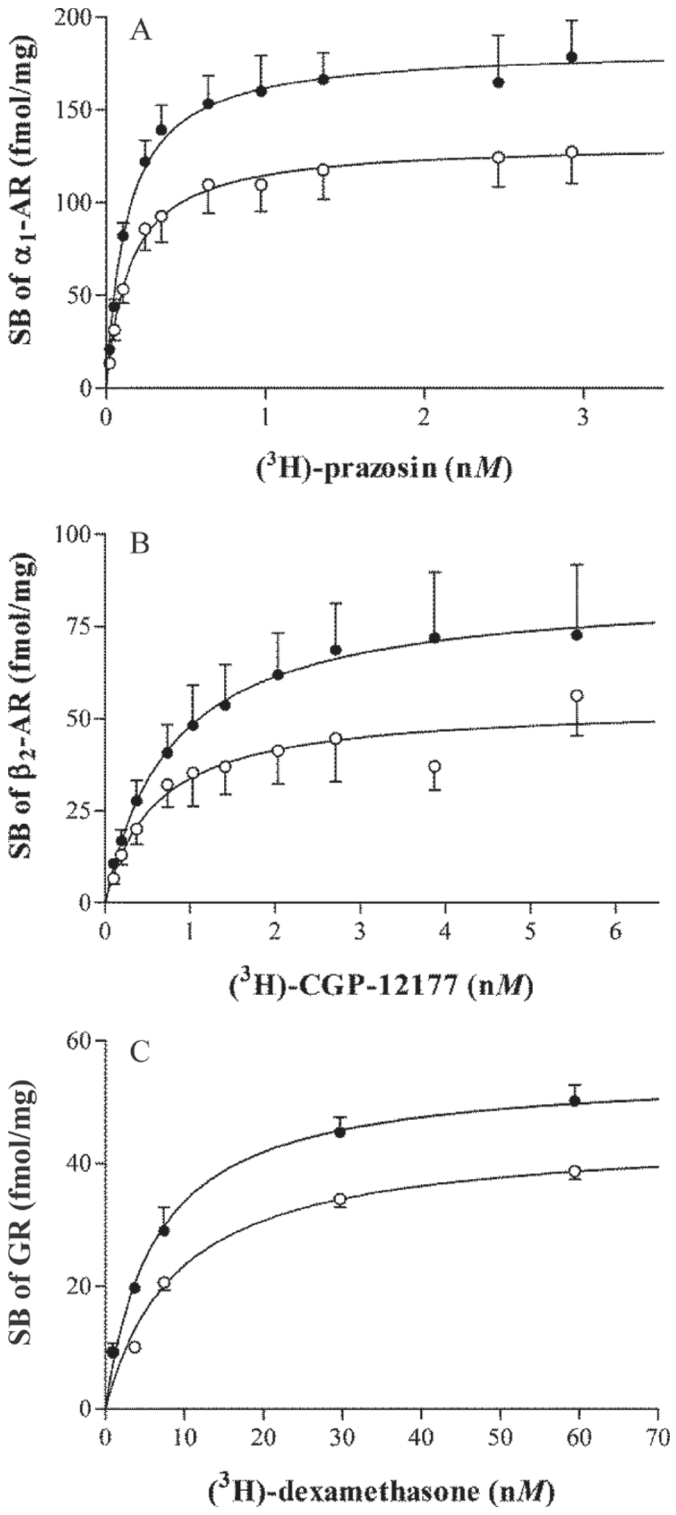

Figure 1. Saturation binding assays of calves fed colostrum (CF; ●) or formula (FF; ○). Specific binding (SB) shown as mean ± SE for each group was determined with increasing concentration of [^{3}H]-prazosin and excess of phentolamine [for α_1-adrenergic receptor (α_1-AR); A], [^{3}H]-CGP-12177 and propranolol [for β_2-adrenergic receptor (β_2-AR); B], and [^{3}H]-dexamethasone and RU 28362 [for glucocorticoid receptor (GR); C]. In each figure, individual plots represent means of 7 calves.

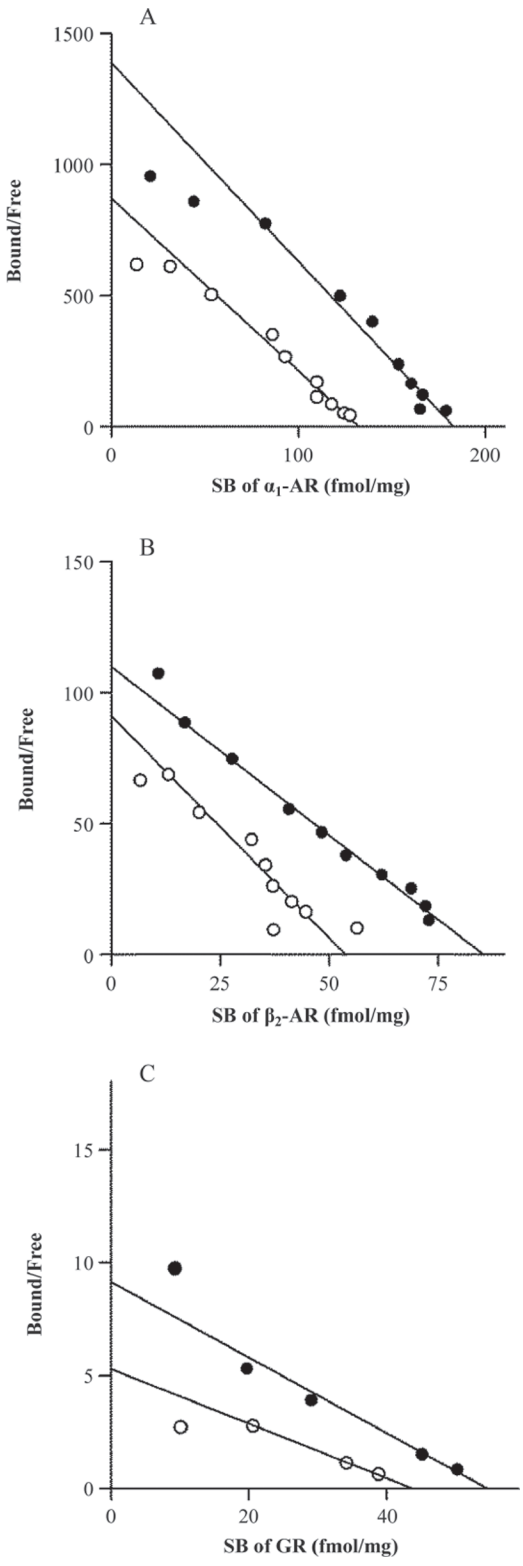

Figure 2. Scatchard plots created from binding studies of [^{3}H]-prazosin [for α_1-adrenergic receptor (α_1-AR); A], [^{3}H]-CGP-12177 [for β_2-adrenergic receptor (β_2-AR); B], and [^{3}H]-dexamethasone [for glucocorticoid receptor (GR); C] in calves fed colostrum (CF; ●) or formula (FF; ○). In each figure, individual plots represent means of 7 calves. SB = specific binding.

inadequate colostrum intake in calves immediately after birth affects neonatal gut development and results in impaired energy metabolism, particularly glucose absorption, resulting in low plasma glucose concentrations (Steinhoff-Wagner et al., 2011a; Hammon et al., 2012, 2013). As a consequence, the maturation of the postnatal somatotropic axis is delayed and leptin status is reduced in calves with insufficient colostrum supply (Hammon and Blum, 1997; Sauter et al., 2003; Blum et al., 2005). In the present study, we observed reduced plasma concentrations of IGF-I, leptin, T_3, and T_4 as well as reduced plasma insulin, but elevated plasma cortisol concentrations, as was shown in other experiments (Hammon and Blum, 1998; Rauprich et al., 2000; Steinhoff-Wagner et al., 2011a), indicating an impaired energy status in calves fed no colostrum but a milk replacer with comparable energy content (Brockman and Laarveld, 1986; Hammon et al., 2012, 2013). Greater plasma concentrations of leptin may point to larger fat depots in colostrum-fed calves (Reidy and Weber, 2000; Ingvartsen and Boisclair, 2001; Blum et al., 2005) and greater IGF-I plasma concentrations accentuate stimulation of anabolic processes in calves after feeding colostrum (Hammon and Blum, 1997; Sauter et al., 2003; Hammon et al., 2012). Decreasing concentrations of T_3 and T_4 during the first week of life has been reported previously in calves, but diet effects on T_3 and T_4 status in neonatal calves are inconsistent (Grongnet et al., 1985; Hammon and Blum, 1998; Rauprich et al., 2000).

The importance of these hormones to energy status in neonatal calves was underlined by the close relationship of these hormones with LGC on d 4 of life. Furthermore, regulation of neonatal thermogenesis might be affected in formula-fed calves, because thyroid hormones and leptin, in addition to catecholamines, which were not measured in the current study, are the primary regulators of neonatal thermogenesis (Reidy and Weber, 2000; Zimmermann-Belsing et al., 2003; Hammon et al., 2012). There is an obvious lack of promoting postnatal growth and maturation in calves fed milk with the same nutrient content as colostrum but no bioactive factors, such as growth factors, hormones, cytokines, and other peptides that support postnatal development and maturation (Blum, 2006; Gauthier et al., 2006; Blum and Baumrucker, 2008). The huge increase in plasma triacylglyceride concentrations in FF calves on d 4 was surprising, because fat intake was the same between groups, in contrast to previous findings where plasma triacylglycerides increased much more after colostrum than formula feeding (Rauprich et al., 2000; Hammon et al., 2012). However, fat in the present formula was from vegetable fat and based on palm and coconut oil, whereas in previous studies, milk

Table 3. Maximal binding capacity (B_{max}) and dissociation constant (K_d) of [^{3}H]-prazosin [for α_1-adrenergic receptor (α_1-AR)], [^{3}H]-CGP-12177 [for β_2-adrenergic receptor (β_2-AR)], and [^{3}H]-dexamethasone [for glucocorticoid receptor (GR)] derived from saturation binding assays in liver tissue of neonatal calves fed either colostrum (CF) or formula (FF)[1]

	Diet			
Item	CF	FF	SEM	*P*-value
B_{max} (fmol/mg of protein)				
α_1-AR	185.21	135.69	19.10	0.09
β_2-AR	85.02	52.72	14.65	0.15
GR	50.81	39.29	3.64	0.05
K_d (n*M*)				
α_1-AR	0.15	0.15	0.02	0.88
β_2-AR	0.68	0.76	0.07	0.40
GR	3.61	4.81	0.84	0.34

[1]Data are presented as LSM and SEM.

fat was used as the fat component in formula (Rauprich et al., 2000). Formula-fed calves had greater plasma albumin concentrations than colostrum-fed calves. However, concentration differences between groups were seen before first feeding and were not the result of protein absorption (e. g., lactalbumin, which was part of the protein fraction in the formula) in FF calves, because plasma albumin concentrations did not increase after formula feeding on d 1 of life. We assume that almost no lactalbumin was absorbed by the intestine in FF calves during first day of life, as was the case for IgG in CF calves, as indicated by elevated total protein concentrations in the blood plasma of CF calves but not FF calves (Steinhoff-Wagner et al., 2011a).

Catecholamines and the adrenergic system play a pivotal role in perinatal maturation of metabolic processes in the liver related to energy supply (e.g., eGP; Apatu and Barnes, 1991; Fowden and Forhead, 2011) and FA oxidation (Olubadewo and Heimberg, 1993) in neonates, including calves (Hammon et al., 2012, 2013). Plasma concentrations of adrenaline and noradrenaline are elevated around birth and support metabolic adaptation for postnatal life (Eliot et al., 1981; Richet et al., 1985; Hume et al., 2005). Effects of adrenaline and noradrenaline are mediated by α- and β-AR, respectively, which are present in liver cell membranes (Rizza et al., 1980; Schmelck and Hanoune, 1980). In the liver, the main subtypes of AR are α_1-AR and β_2-AR, and the first one is the main mediator of hepatic catecholamine action (Aggerbeck et al., 1980; Goodhardt et al., 1984; Sulakhe et al., 1988). Recent investigations in the liver of neonatal calves indicated α_{1A}- and β_2-AR as the dominant subtypes (Carron et al., 2005a,b; Ontsouka et al., 2006). In the present study, we confirmed the existence of these 2 adrenergic subtypes in the liver of neonatal calves. Binding affinities (K_d values) for each, α_1- and β_2-AR, were comparable between feeding groups and Scatchard plots indicated single receptor binding types for α_1- and β_2-AR. In addition, we found greater B_{max} for α_1- than for β_2-AR in our calves, which may indicate the higher importance of α_1-AR for adrenergic regulation of hepatic energy metabolism in neonatal calves (Aggerbeck et al., 1980; Goodhardt et al., 1984).

On the other hand, feeding effects on B_{max} of AR subtypes were rare, showing a trend for greater number of α_1-AR in colostrum-fed calves, but no significant differences between feeding groups with respect to B_{max} of hepatic β_2-AR, which supports previous findings (Carron et al., 2005a). The trend for greater B_{max} of α_1-AR in colostrum-fed calves might be in accordance with the increasing importance of α_1-AR for mediating sympathetic regulation of hepatic energy metabolism in the neonate, as seen for rats (Schmelck and Hanoune, 1980; Morgan et al., 1983; Huff et al., 1991), but correlations

Table 4. Hepatic mRNA abundance ($\log_2$) of α_1- and β_2-adrenergic receptors and glucocorticoid receptor as well as of enzymes and transcription and growth factors involved in lipid metabolism[1]

	Group[3]			
Item[2]	CF	FF	SEM	*P*-value
ADRA1	0.33	0.54	0.08	0.08
ADRB2	0.08	0.07	0.02	0.73
NR3C1	1.04	1.28	0.21	0.45
CPT1A	0.98	1.46	0.28	0.25
ACADVL	1.19	0.76	0.25	0.25
ACADM	21.94	18.31	4.07	0.54
ACSL	11.38	13.15	2.66	0.65
PPARA	8.57	10.75	1.83	0.45
IGF1	0.34	0.34	0.12	0.99

[1]Values are shown as LSM and SEM.

[2]*ADRA1* = α_1-adrenergic receptor; *ADRB2* = β_2-adrenergic receptor; *NR3C1* = glucocorticoid receptor; *CPT1A* = carnitine palmitoyltransferase 1α; *ACADVL* = acyl-CoA dehydrogenase very long chain; *ACADM* = acyl-CoA dehydrogenase medium chain; *ACSL* = acyl-CoA synthetase long chain; *PPARA* = peroxisome proliferator activated receptor α.

[3]CF = colostrum-fed calves; FF = formula-fed calves.

of α_1-AR with parameters of hepatic energy metabolism (e.g., eGP) were barely detectable in our study. Both α_1- and β_2-AR are involved in hepatic regulation of eGP (Schmelck and Hanoune, 1980; Goodhardt et al., 1984; Apatu and Barnes, 1991) and probably also in regulation of FA oxidation (Schulze et al., 1986). However, different diets did not affect eGP on d 3 of life in our calves (Steinhoff-Wagner et al., 2011a) and no correlations of α_1-AR binding density and eGP could be observed. Surprisingly, B_{max} of β_2-AR was negatively related to eGP and glucose oxidation, which might indicate minor importance of hepatic β_2-AR on endogenous glucose supply.

In contrast to previous results on gene and protein expression of AR, mRNA abundance of *ADRA1* and *ADRB2* did not correlate with B_{max} of α_1- and β_2-AR, respectively (Carron et al., 2005a,b; Ontsouka et al., 2006). Furthermore, *ADRA1* mRNA abundance tended to be greater in formula-fed calves. The reason for the missing relationships between binding densities and mRNA abundance of AR are presently not known, but liver tissue from calves in the current study was taken 2 h after feeding, whereas liver tissue in previous studies was from fasted calves (Carron et al., 2005a,b; Ontsouka et al., 2006). Therefore, feed intake might have an immediate effect on hepatic gene expression of AR, whereas the feeding response on membrane-bound AR density might be delayed. Interestingly, *ADRA1* mRNA abundance indicated some positive relationship to parameters of hepatic FA oxidation and *IGF1* mRNA abundance, but negative relationship to LGC and plasma leptin concentrations. Therefore, *ADRA1* mRNA abundance seems to be involved in regulation of metabolic status in the liver and may be related to glycogenolysis (Goodhardt et al., 1984), although this was not the case for α_1-AR binding density. Because leptin affects 5-AMP-activated protein kinase and FA oxidation in the liver by stimulating the hepatic α_1-AR, negative feedback regulation of *ADRA1* mRNA might occur by the leptin status in the liver of neonatal calves. Therefore, adrenergic effects on hepatic FA oxidation might be mediated by α_1- and β_2-AR as well as plasma leptin concentrations (Lafontan and Berlan, 1993; Miyamoto et al., 2012).

As mentioned above, glucocorticoid concentrations are elevated in blood plasma during the perinatal period (Cabello, 1979), glucocorticoids promote metabolic adaptation to postuterine life (Fowden et al., 1998; Schmidt et al., 2004; Hammon et al., 2012), and plasma cortisol concentrations in neonatal calves are affected by diet (Nightengale and Stott, 1981; Hammon and Blum, 1998; Rauprich et al., 2000). In the present study, formula versus colostrum feeding influenced GR density in liver, indicating a greater number of GR in colostrum-fed calves. The importance of hepatic GR for postnatal energy metabolism in these calves was emphasized by the close relationship of GR binding capacity with PEPCK enzyme activity and plasma concentrations of glucose, NEFA, insulin, and IGF-I. Obviously, hepatic GR are involved in regulation of postnatal energy status in neonatal calves. Sufficient energy supply and utilization are a precondition for maturation of the postnatal somatotropic axis, which is promoted by glucocorticoid action (Fowden et al., 1998; Breier et al., 2000; Sauter et al., 2003) and this is probably mediated by the hepatic GR in neonatal calves.

Although the application of dexamethasone as potent glucocorticoid analog did not stimulate PEPCK activity and eGP in calves (Hammon et al., 2003; Scheuer et al., 2006), hepatic GR activation is important for stimulation of PEPCK enzyme activity (Pilkis and Granner, 1992) and eGP (Edgerton et al., 2006). The GR mediates glucocorticoid stimulation of *PEPCK* gene expression in liver, which is an important regulator for PEPCK enzyme activity (Pilkis and Granner, 1992; Hanson and Reshef, 1997; Rose et al., 2010). Although we have not found a significant relationship between GR density and mRNA abundance of *PCK1* and *PCK2*, GR binding density may contribute to the regulation of PEPCK in the liver of neonatal calves, as we have found a close relationship between GR binding density and PEPCK enzyme activity.

Interestingly, GR binding density is related to mRNA abundance of *ACADM*, which is involved in FA oxidation, and hepatic mRNA abundance of *NR3C1* is also related to factors involved in FA oxidation (*ACADM*, *CPT1A*, and *PPARA*) and energy status (*IGF1*) in the liver. Carnitine palmitoyltransferase 1α mediates the entrance of NEFA into the mitochondria and, therefore, is rate limiting for FA oxidation. Less carnitine palmitoyltransferase activity results in a greater accumulation of FA in the liver. Additionally, *CPT1A* is a direct target gene of *PPARA*, which is a major regulator of hepatic FA oxidation (Mandard et al., 2004). However, it is arguable whether the positive relationship of *NR3C1* with parameters of FA oxidation implies stimulation of FA oxidation by GR activation in calves, because stimulation of hepatic GR leads to inhibition of hepatic FA oxidation in mice (Lettéron et al., 1997; Rose et al., 2010). Furthermore, neither GR density nor mRNA abundance of *NR3C1* were related to LFC in our study, which may indicate less importance of the GR for regulation of FA oxidation and fat accumulation in neonatal calves. Interestingly, at the transcriptional level, *CPT1A* and *PPARA* were correlated with *PC*, *PCK2*, and *G6PC* as well as *ACADM*, underpinning the

coordinated regulation of FA oxidation and gluconeogenesis in the liver, where gene expression of *ADRA1* and *NR3C1* might be involved.

In conclusion, the endocrine metabolic status in neonatal calves either fed colostrum or a milk-based formula with the same nutrient content as colostrum was different; however, many fewer bioactive factors exist, such as growth factors and hormones. Saturation binding studies on α_1- and β_2-AR as well as GR in the liver revealed a strong diet effect on GR density, but diet effect on α_1-AR density was less accentuated. These results confirm the involvement of hepatic GR and AR in maturation of postnatal energy metabolism and underline the importance of colostrum feeding as well as the glucocorticoid and adrenergic system for postnatal adaptation of glucose and lipid metabolism in calves.

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