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Activities of γ-butyrobetaine dioxygenase and concentrations of carnitine in tissues of pigs

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ABSTRACT

In contrast to other species, less is known about carnitine homeostasis in the pig. This study was performed to yield information about the site of carnitine synthesis and carnitine concentrations in various tissues of pigs (*Sus scrofa*). We found that among several pig tissues, a considerable activity of γ-butyrobetaine dioxygenase (BBD), the last enzyme of carnitine synthesis, exists, like in humans and several other species, only in liver and kidney. Activity of that enzyme in liver and kidney was lower at birth than in the subsequent weeks of life. Highest carnitine concentrations were found in skeletal muscle and heart. Carnitine concentrations in plasma, liver and kidney at birth were higher than in the subsequent weeks of life in spite of the low BBD activity at birth. In conclusion, this study shows that liver and kidney are the major sites of carnitine synthesis and that neonatal pigs do not have an insufficient carnitine status.

1. Introduction

Carnitine (L-3-hydroxy-4-N-N-N-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable functions in intermediary metabolism (Steiber et al., 2004). All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis (Hoppel and Davis, 1986; Rebouche and Seim, 1998). Carnitine biosynthesis involves a complex series of reactions. Lysine in protein peptide linkages provides the carbon backbone of carnitine. It undergoes methylation of the ε-amino group to yield trimethyllysine, which is released upon protein degradation. The released trimethyllysine is further oxidised to γ-butyrobetaine which is then hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine (Vaz and Wanders, 2002). Carnitine produced in tissues expressing an active BBD is secreted into the blood and taken up into tissues by novel organic cation transporters (OCTN), particularly OCTN2 which is the most important carnitine transporter (Tamai et al., 2000; Lahjouji et al., 2001).

Tissue distribution of BBD is different between various mammalian species. In all mammals studied so far, BBD activity has been found in the liver (Vaz and Wanders, 2002). In some species such as in humans, cats, cows, hamsters, rabbits or Rhesus monkeys, BBD activity has been detected also in the kidney; in these species, activity of BBD in the kidney is even higher than in the liver (Vaz and Wanders, 2002). In contrast, in several other species such as Cebus monkeys, sheep, dogs, guinea pigs, mice and rats, BBD is not, or only at very low activity, present in the kidney (Vaz and Wanders, 2002). In humans, BBD activity has been also found in the brain which is in contrast to other species (Rebouche and Engel, 1980). In rats, one study detected BBD activity in testis and epididymis (Carter et al., 1987), which could, however, not be confirmed by another study (Galland et al., 1999). In sheep, BBD activity was also observed in muscle (Erfle, 1975). There does not appear to be any evolutionary pattern with respect to the activity of BBD in tissues, since even very closely related species, like the Rhesus and Cebus monkeys, exhibit a different pattern.

In contrast to various other animal species, less is known about carnitine metabolism in the pig. For instance, the site of carnitine biosynthesis in pigs has not yet identified. Moreover, there is also less information about carnitine concentrations in tissues of pigs. In humans carnitine concentrations are highest in skeletal muscle which is regarded as a carnitine storage. Concentration of carnitine in muscle (2000–4000 nmol/g wet weight) is as much as 100times higher than that in plasma (Bertoli et al., 1981; Moorthy et al., 1983; Angelini et al., 1992). Organs such as kidney, liver and brain contain intermediate levels of 300–1000 nmol/g wet weight (Moorthy et al., 1983; Angelini et al., 1992). It is actually unclear whether a similar pattern of tissue carnitine concentrations exists also in pigs.

In humans, activity of BBD in liver is particularly low at birth (Rebouche and Engel, 1980). Moreover, it has been shown that infants fed a formula without supplemented carnitine have low plasma carnitine concentration (Olson et al., 1989). Therefore, it has been suggested that carnitine is an essential nutrient in the newborn (Borum, 1981; Penn et al., 1981). Some studies suggested that pigs have also an insufficient carnitine status at birth (van Kempen and Odle, 1995; Penn et al., 1997; Heo et al., 2000). Therefore, the neonatal

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pig has been suggested as model to study human neonatal carnitine metabolism (Baltzell et al., 1978; Penn et al., 1997). We are however not aware of any study which investigated the carnitine status of neonatal pigs relative to older pigs.

The aim of this study was to gain more insight into carnitine homeostasis in the pig. First, we intended to identify the sites of carnitine biosynthesis in pigs. Therefore, we determined mRNA and protein concentration and activities of BBD in various tissues of pigs. Second, to characterize the tissue carnitine concentration pattern in pigs, we determined carnitine concentrations in various tissues. Third, to find out whether newborn pigs indeed have a low carnitine status at birth, we determined concentrations of carnitine in various tissues of pigs at birth and in the subsequent weeks of life. In order to investigate the hypothesis that carnitine biosynthesis rate is particularly low at birth, we also determined the activity of BBD and concentrations of γ-butyrobetaine, the precursor of carnitine, in pig tissues of pigs at birth and in the subsequent weeks. These studies should also be helpful to characterize the carnitine homeostasis in the pig compared to humans and other mammalian species.

2. Materials and methods

All experimental procedures described followed established guidelines for the care and use of laboratory animals according to law on animal welfare and were approved by the local veterinary office [Halle (Saale), Germany].

2.1. Animals and diets

In order to identify tissues containing BBD, we used four pigs (*Sus scrofa*) (one female, two castrates, one uncastrated male of a crossbred race [(German Landrace × Large White) × Pietrain] with a body mass between 60 and 70 kg. These pigs were fed ad-libitum a nutritionally adequate standard pig diet containing 13.4 MJ metabolizable energy and 170 g crude protein/kg. The diet had a low native carnitine concentration (<5 mg/kg).

In order to investigate the effect of age on activity of BBD and concentrations of carnitine and precursors in plasma and tissues, we used litters of five crossbred sows [Large White × German Landrace × Hermitage) × Pietrain]. During pregnancy, these sows received a standard lactation diet for sows containing 13.4 MJ metabolizable energy and 175 g crude protein/kg. The carnitine concentration of this diet was below 5 mg/kg. Immediately after birth, the litters of those sows were standardized to eight (four male, four female) pigs/litter. From day 11 until weaning, the piglets were offered a creep feed for ad-libitum consumption which contained 15.6 MJ metabolizable energy and 200 g crude protein/kg diet; the carnitine concentration was 35 mg/kg. The piglets were weaned at day 28 and were offered the creep feed for ad libitum consumption until day 35. Thereafter, they were switched to a nutritionally adequate piglet diet which contained 13.7 MJ metabolizable energy and 185 g crude protein/kg. The native carnitine concentration of this diet was below 5 mg/kg. Immediately after birth and at the end of each following week, one piglet/litter was removed from each of the five sows and used for the collection of samples.

Concentrations of crude protein in the diets were analysed according to the official German VDLUFA methodology (Bassler and Buchholz, 1993). The metabolisable energy of the diet was calculated as recommended by the German Nutrition Society (Gesellschaft für Ernährungsphysiologie, 2006).

2.2. Sample collection

The animals were anaesthesised and exsanguinated. Blood samples were collected into heparinised polyethylene tubes. In the first experiment, liver, kidneys, heart, proximal segments of small intestine (duodenum) and colon and samples from m. *longissimus dorsi*, brain, lung and spleen were excised. In the uncastrated male animal, additionally one testis and epididymis were prepared. In the second experiment, liver, kidneys, heart and samples from m. *longissimus dorsi* and m. *semimembranosus* were excised from each animal. Plasma was obtained in each experiment by centrifugation of the blood samples (1100 *g*, 10 min, 4 °C). Plasma and tissue samples were stored at −20 °C.

2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated from tissue samples using Trizol™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Total RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Synthesis of cDNA and determination of mRNA abundance by RT-PCR with real-time detection (Rotorgene 6000, Corbett Research, Australia) using Sybr Green I was performed as recently described in detail (Ringseis et al., 2007). For absolute quantification of mRNA abundance of BBD and GAPDH standard curves were generated with purified PCR products of BBD and GAPDH which were obtained by extraction of cut ethidium bromide-stained bands following 2% agarose gel electrophoresis by MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Ct values for each amplification curve were obtained using Rotorgene Series Software 1.7 (Corbett Research, Australia). Quantification of double-stranded DNA concentration of purified PCR products was performed using the PicoGreen DNA Quantitation Kit (Molecular Probes) and a spectrofluorometer (excitation: 480 nm, emission: 520 nm). For normalization purposes, the copy number of the housekeeping gene GAPDH served as an independent internal control. Sequences of gene-specific primers obtained from Operon (Köln, Germany) were as follows (forward, reverse; NCBI Genbank): BBD (5′-GTG CCG AAA GCT CAA GGA AAA A-3′, 5′-CTC TGC CGG CCG TGA AGT AAC-3′; partial sequence according to Ruan et al. (2007) and GAPDH (5′-AGG GGC TCT CCA GAA CAT CAT CC-3′, 5′-TCG CGT GCT CTT GCT GGG GTT GG-3′; AF017079).

2.4. Immunoblot analysis of BBD

For immunoblotting, homogenates of all tissues were prepared by homogenising tissue aliquots in 10 mM 3-morpholinepropansulfonic acid buffer (pH 7.4) containing 0.9% (w/v) sodium chloride, 10% (w/v) glycerol, and 5 mM dithiothreitol, and protein concentrations were determined by the Bradford method (Bradford, 1976). The brain was not considered for immunoblot analysis, because this tissue could not be completely homogenised using the above mentioned buffer. Equal amounts of protein (50 µg) were electrophoresed by 12.5% SDS-PAGE, and transferred to a nitrocellulose membrane (Pall, Pensacola, USA). The membranes were blocked overnight at 4 °C in 5% skim milk in Tris-buffered saline containing 0.2% Tween (TBS-T), and then incubated with a 1:500 dilution of a mouse monoclonal anti-BBD primary antibody (ab56350; Abcam, Cambridge, UK) for 2 h at room temperature. Membranes were washed with TBS-T, and incubated with a HRP conjugated secondary antibody anti-mouse IgG (Sigma-Aldrich, Taufkirchen, Germany) for 1.5 h at room temperature. Afterwards, blots were washed again, and developed using ECL Plus (Western Blotting Detection Reagents, GE Healthcare Europe GmbH, Freiburg, Germany). For normalisation purposes, membranes were also incubated with a mouse monoclonal anti-GAPDH primary antibody (ab8245; Abcam, Cambridge, UK). The signal intensities of specific bands (BBD and GAPDH) were detected with a Bio-Imaging system (Bio-Imaging Systems, F-ChemiBIS 3.2 M, biostep GmbH, Jahnsdorf, Germany) and quantified using TotalLab TL100-Quick Start analysis software (nonlinear dynamics). The normalised protein concentration was calculated as the ratio of the band intensities of BBD and GAPDH.