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EVIDENCE FOR CATABOLIC PATHWAY OF PROPIONATE METABOLISM IN CNS: EXPRESSION PATTERN OF METHYLMALONYL-CoA MUTASE AND PROPIONYL-CoA CARBOXYLASE ALPHA-SUBUNIT IN DEVELOPING AND ADULT RAT BRAIN

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Abstract—Methylmalonyl-CoA mutase (MCM) and propionyl-CoA carboxylase (PCC) are the key enzymes of the catabolic pathway of propionate metabolism and are mainly expressed in liver, kidney and heart. Deficiency of these enzymes leads to two classical organic acidurias: methylmalonic and propionic aciduria. Patients with these diseases suffer from a whole spectrum of neurological manifestations that are limiting their quality of life. Current treatment does not seem to effectively prevent neurological deterioration and pathophysiological mechanisms are poorly understood. In this article we show evidence for the expression of the catabolic pathway of propionate metabolism in the developing and adult rat CNS. Both, MCM and PCC enzymes are co-expressed in neurons and found in all regions of the CNS. Disease-specific metabolites such as methylmalonate, propionyl-CoA and 2-methylcitrate could thus be formed autonomously in the CNS and contribute to the pathophysiological mechanisms of neurotoxicity. In rat embryos (E15.5 and E18.5), MCM and PCC show a much higher expression level in the entire CNS than in the liver, suggesting a different, but important function of this pathway during brain development.

Key words: methylmalonic aciduria, propionic aciduria, MCM, MUT, PCCA, developing brain.

Methylmalonyl-CoA mutase (MCM) and propionyl-CoA carboxylase (PCC) are the key enzymes of the catabolic pathway of the amino acids isoleucine, valine, methionine and threonine, as well as of odd-chain fatty acids and the side chain of cholesterol.

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Abbreviations: AdoCbl5, deoxyadenosylcobalamin; BCAA, branched chain amino acids; DEPC, diethylpyrocarbonate; GFAP, glial fibrillary acidic protein; HRP, horse radish peroxidase; ISH, *in situ* hybridization; MBP, myelin basic protein; MCM, methylmalonyl-CoA mutase; MMA, methylmalonic acid; NeuN, neuronal nuclei protein; PCC, propionyl-CoA carboxylase; PCCA/PCCα, propionyl-CoA carboxylase alpha-subunit; PCCB/PCCβ, propionyl-CoA carboxylase beta-subunit; PFA, paraformaldehyde; TCA, tricarboxylic acid.

MCM (EC, 5.4.99.2) is a mitochondrial enzyme first identified in 1955 in sheep kidney (Flavin et al., 1955) and rat liver (Katz and Chaikoff, 1955) that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA. It requires 5′-deoxyadenosylcobalamin (AdoCbl) as a cofactor. MCM is encoded by the *MUT* gene (MIM *609058) which maps to chromosome 6p12-21.2 (Ledley et al., 1988), and encodes a 750 amino acid protein (Nham et al., 1990). After cleavage of the mitochondrial leader sequence (32 amino acids at the N-terminus), the remaining 79 kDa subunit assembles into a homodimer that binds two molecules of AdoCbl, thus forming the active holoenzyme (Kolhouse et al., 1980; Fenton et al., 1984). MCM mRNA and enzyme activity were studied in different adult murine tissues, showing a rank-order correlation between the relative level of enzyme activity and mRNA in each tissue with the highest levels in kidney, followed by intermediate levels in heart, liver, ovary, brain and muscle and low levels in lung and spleen (Wilkemeyer et al., 1993). Alterations of the *MUT* gene cause methylmalonic aciduria (MIM #251000). Mutations in *MUT* may cause partial (*mut*$^-$) or complete (*mut*^{0}) enzyme deficiency. Patients with methylmalonic aciduria typically present in the newborn period with ketoacidosis, lethargy, vomiting and failure to thrive which can be lethal if untreated (Matsui et al., 1983). Late onset and mild phenotypes have been observed, particularly in *mut*$^-$ patients. There is a good correlation between residual enzyme activity and severity of the clinical phenotype (Shevell et al., 1993).

PCC (EC, 6.4.1.3) catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA. It is a heterocopolymer of α (PCCα) and β (PCCβ) subunits (Lamhonwah et al., 1986) with a configuration of either $\alpha4\beta4$ or $\alpha6\beta6$ (Campeau et al., 1999). The α subunit (72 kDa) contains a binding site for the cofactor biotin, an ATP binding site and a biotin carboxylating site (Perez et al., 2003). It is encoded by the *PCCA* gene (MIM *23200) on chromosome 13q32 (Kennerknecht et al., 1992). The β subunit (56 kDa) is encoded by the *PCCB* gene (MIM *232050) on chromosome 3q13.3-q22 (Yang-Feng et al., 1985) and carries the carboxybiotin and propionyl-CoA binding sites. Deficient PCC activity leads to propionic aciduria (MIM #606054). Cells from patients with mutations in *PCCA* fall into complementation group pccA. Cells from patients with mutations in *PCCB* fall into two complementation subgroups, pccB and pccC. Mutations in the pccB subgroup occur in the N-terminus of the β subunit, which includes the

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carboxybiotin-binding site, whereas mutations in the pccC subgroup occur in the C-terminus (Fenton et al., 2001). Clinical presentation is similar to that seen in methylmalonic aciduria.

Neurological symptoms are frequently found in patients with methylmalonic and propionic acidurias. They may occur acutely, such as "metabolic stroke" and acute encephalopathic crises characteristically involving the basal ganglia (Matsui et al., 1983; Heidenreich and Natowicz, 1988; van der Meer et al., 1994). However, neurological deterioration may progress over many years, resulting in cerebral or cerebellar atrophy, basal ganglia injury, and white matter disease (Brismar and Ozand, 1994; Harting et al., 2008). Due to the involvement of grey and white matter in various brain regions, the range of neurological presentations of affected individuals is wide, including developmental delay, motor dysfunction (e.g. muscular hypotonia, spasticity, dystonia, chorea, ataxia), cognitive dysfunction (e.g. mental retardation, delayed speech development, behavioral problems), psychiatric disease, epilepsy, and microcephaly (Nicolaides et al., 1998; Horster et al., 2007).

Three cerebral pathomechanistic concepts have been proposed for neurological damage in these organic acidurias:

The "toxic metabolite hypothesis": Methylmalonate (MMA, the key metabolite of methylmalonic aciduria) was first considered as the main neurotoxic metabolite, whereas other studies suggested toxic effects of propionyl-CoA and 2-methylcitrate (Okun et al., 2002; Kölker et al., 2003). MMA has structural similarities with known inhibitors of respiratory chain complex II and was thought to be a mitochondrial toxin (Wajner and Coelho, 1997). Toxic effects of MMA on primary neuronal cultures and in rats after intra-striatal administration have been shown and could be prevented by succinate, N-methyl-D-aspartate receptor antagonists and antioxidants (Okun et al., 2002).

Synergistic inhibition of mitochondrial energy metabolism: MMA loading in cultured rat striatal neurons results in intracellular accumulation not only of MMA, but also of 2-methylcitrate and malonate (Okun et al., 2002). Thus, impairment of energy metabolism might be mediated by a synergistic inhibition of tricarboxylic acid (TCA) cycle and mitochondrial respiratory chain by 2-methylcitrate, MMA and propionyl-CoA (Kölker et al., 2003). Oxidative phosphorylation studies in muscle tissue of propionic aciduria patients showed severely decreased activity of complexes I–IV and mtDNA depletion (Schwab et al., 2006).

Dicarboxylic acids and the "trapping hypothesis": The blood–brain barrier has a limited transport capacity for decarboxylates (Sauer et al., 2006). It has been hypothesized that brain-generated decarboxylates might be trapped in CNS and cause neurodegeneration in organic acidurias. It was also assumed that MMA might interfere with the transport of decarboxylates between neurons and astrocytes (Kölker et al., 2006). Consistent with this hypothesis, Kaplan et al. (2006) report on a long-term (9 years) outcome of a liver transplanted patient with methylmalonic aciduria, in whom plasma and urine MMA levels decreased significantly after transplantation, whereas concentrations of MMA and 2-methylcitrate remained high in CSF, suggesting a primary metabolism of MMA in brain.

Knowledge on the underlying mechanisms of neurological manifestations in methylmalonic and propionic aciduria is still poor. Effective treatment strategies to prevent neurological deterioration are still lacking. As a consequence, the risk of severe disability, reduced life expectancy and poor quality of life is very high in affected individuals.

In this article we show the cell-specific expression patterns of MCM and PCCα in the developing and adult rat CNS in order to test the hypothesis that the catabolic pathway of propionate metabolism is expressed in CNS and could contribute to the neuropathogenesis in methylmalonic and propionic aciduria.

EXPERIMENTAL PROCEDURES

Preparation of rat tissues

Animal experiments were approved by the Cantonal Veterinary Office and the Cantonal Committee for Animal Experiments, in accordance with the State Veterinary Office of the Canton Vaud, the Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Experiments were designed to keep the animal number at a minimum and care was taken to minimize suffering. Pregnant female rats (Sprague–Dawley, 300 g; Harlan, The Netherlands) were sacrificed by decapitation. Embryos (stages: embryonic days 15.5 and 18.5, E15.5 and E18.5 respectively) were immediately removed from uterus, rinsed in ice-cold diethylpyrocarbonate (DEPC)-treated PBS and fixed for 15 h at room temperature in 4% paraformaldehyde (PFA) in DEPC-treated PBS. Subsequently, embryos were cryoprotected at 4 °C in 12% and 18% sucrose in DEPC-treated PBS for 18 h and 24 h respectively, then embedded in cryoform (O.C.T. compound tissue-tek, Digitana, Switzerland) and frozen in liquid nitrogen-cooled isopentane. Embryos were stored at −80 °C until used for cryosections. For adults, female Sprague–Dawley rats were sacrificed by decapitation, and their brain and liver immediately dissected out and rinsed in ice-cold DEPC-treated PBS. Adult brains were either immediately embedded in cryoform, frozen in liquid nitrogen-cooled isopentane and stored at −80 °C until used for cryosections, or frozen in liquid nitrogen and stored at −80 °C for protein extraction. Adult livers were immediately frozen in liquid nitrogen and stored at −80 °C for protein extraction.

In situ hybridization

Partial or complete open reading frame cDNAs of rat *Mut* (encoding MCM, nucleotides 256-2502, Gene Bank XM_001067239.1) and *Pcca* (encoding PCCα, nucleotides 33-2304, Gene Bank NM_019330) genes were isolated from rat brain and liver mRNA by reverse transcription coupled to PCR. The amplified cDNAs were verified by sequencing, inserted into the XbaI and HindIII cloning sites of the pBluescript II KS$^-$ vector (Stratagene, Agilent Technologies, CA, USA), and used to synthesize antisense and sense digoxigenin-labeled MCM and PCCα riboprobes, as described previously (Braissant et al., 2001b; Braissant, 2004). Adult brain and whole embryo cryosections (16 μm thick) were prepared and analyzed by *in situ* hybridization (ISH) as described (Braissant et al., 2001a, 2005). Briefly, cryosections were postfixed 10 min in 4% PFA in PBS, washed 2×15 min in PBS containing 0.1% fresh DEPC and equilibrated 15 min in 5× SSC. Sections were hybrid-