Subcellular transfer of reducing equivalents in the neuronal and glial compartments of the adult rat brain after portocaval anastomosis and chronic moderate hyperammonemia as detected by (^{13}C, ^{2}H) NMR

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Introduction. Transfer of reducing equivalents from cytosol to mitochondria plays a vital role in the energetics of the adult brain, allowing to reoxidize in the mitochondria the NADH produced in the cytosol during aerobic glycolysis. The process occurs mainly through the operation of the malate-aspartate shuttle, a mechanism involving the exchange of glutamate (glu) and α-ketoglutarate (α-kg) between cytosol and mitochondria. Pathological limitations in oxygen supplies as in hypoxia or ischemia, or dysfunction in these transporters, lead to limitations in the transfer of reducing equivalents and result in reduced cytosolic redox state and lactate accumulation. Despite its importance, protocols to investigate the turnover of reducing equivalents *in vivo* were not available until very recently. We developed a novel ^{13}C NMR procedure, using a double ^{2}H and ^{13}C labeling technique that allows monitoring the traffic of glu molecules between mitochondria and cytosol of perfused liver and intact brain. The method is based on the fact that fast kinetics of single deuteration in the H3 hydrogens of glu or glutamine (gln) reflects the turnover of the cytosolic glu pool, while the slower double deuteration in H3, H3' may then proceed from the turnover of the mitochondrial pool, during the *de novo* formation of [2-^{13}C]glu or gln in the TCA cycle and associated anaplerotic reactions. It then becomes possible to investigate alterations in the traffic of glu molecules through mitochondria and cytosol by determining the relative proportions of monodeuterated H3 and doubly deuterated H3,H3' glu molecules. These distinct patterns of deuteration in [2-^{13}C]glu and [2-^{13}C]gln can be easily distinguished by ^{13}C NMR spectroscopy since perprotonated, H3 monodeuterated and H3,H3'doubly deuterated glu molecules are easily identified by their distinct chemical shifts (1). In this report, we describe the effects of portocaval anastomosis and a moderate hyperammonemia without liver failure on the exchange by ^{2}H of the H3 and/or H3' hydrogens of cerebral [2-^{13}C]glu and [2-^{13}C]gln, in adult rats receiving a [1-^{13}C]glucose infusion (2).

Methods. Well-nourished Wistar rats (n=4) were anesthetized with halothane and an end-to-side portacaval anastomosis was constructed surgically under aseptic conditions using a continuous suture technique. The inferior vena cava and portal vein were clamped for not more than 15 min; after uncampling, the bowel was evaluated for cyanosis. If cyanosis persisted, the animal was sacrificed. Sham operated control rats (n=4) had their portal vein and inferior vena cava clamped for 10 min. After satisfactory surgery, the abdomen was sutured in two layers and rats returned to their individual cages with the same housing conditions. The anastomosis was examined at the moment of sacrifice, the liver was atrophic and the anastomosis was permeable. The changes in body weight following portacaval anastomosis were similar to those usually observed in this well characterized animal model (3). Wistar rats were made hyperammonemic (n=4) by feeding them a diet containing ammonium acetate (20% by weight) for 24 days. Control animals (n=4) received conventional rat chow. In this protocol, the increase of ammonia concentration in blood and brain are similar to those found in patients with liver cirrhosis. Ten days before sacrifice, drinking water was replaced in all the cases by water containing 50% (vol/vol) $^{2}H_2O$. Then, all animals were anesthetized and infused (60 min), with a 0.2M solution of [1-^{13}C]glucose (8 μmol.min^{-1}.100 g^{-1}). At the end of the infusions, brain extracts were prepared and analyzed by high-resolution ^{13}C NMR spectroscopy (125.13 MHz for ^{13}C, 22 °C).

Results. Figure 1 compares the relative proportions of [2-^{13}C], [2-^{13}C, 3-^{2}H] and [2-^{13}C,3,3'-$^{2}H_2$]glu and gln isotopomers in control and dietary hyperammonemic rats with those obtained in sham and portocaval anastomosis operated animals. No significant difference in the deuterated isotopomer populations was detected in the chronic hyperammonemic condition, as compared the significant alterations detected in portocaval shunted animals. In the case of glu, portocaval anastomosis increased the relative contribution of [2-^{13}C]glu, diminished that of [2-^{13}C, 3-^{2}H]glu and decreased to reduced levels the contribution of [2-^{13}C, 3,3'-$^{2}H_2$]glu (Figure 1, dss). Gln followed a similar behavior. Portocaval anastomosis increased the contribution of [2-^{13}C]gln, but decreased significantly the contributions of [2-^{13}C, 3-^{2}H] and [2-^{13}C, 3,3'-$^{2}H_2$]gln. These measurements of subcellular glu turnover as implemented by [^{13}C, ^{2}H] NMR provide an important assessment on the transfer of reducing equivalents between these compartments. The exchange of [2-^{13}C, 3-^{2}H] and [2-^{13}C, 3,3'-$^{2}H_2$]glu, through the mitochondrial membrane, is slow in the timescale of cytosolic isocitrate dehydrogenase or aconitase ^{2}H exchanges. On this basis, it is possible to propose that the steady state level of [2-^{13}C, 3-^{2}H]glu reflects the cytosolic glu concentrations while the steady state level of [2-^{13}C, 3,3'-$^{2}H_2$]glu reflects mainly the mitochondrial glu concentration. A very similar reasoning may be proposed for the H3, H3' deuterations in [2-^{13}C]gln. Since glu and gln represent the neuronal and glial pools of glu during the metabolism of [1-^{13}C]glucose, the deuteration patterns observed in these metabolites may be assigned to processes occurring in neurons and glial cells, respectively. The results obtained in porocaval anastomosis are consistent with a drastic reduction in the transfer of reducing equivalents through the mitochondrial membrane of the neurons and a concomitant decrease of the neuronal TCA cycle activity. In the case of gln, it results in a reduction in the relative contribution of [2-^{13}C, 3-^{2}H]gln and a more pronounced reduction in the relative contribution of [2-^{13}C, 3,3'-$^{2}H_2$]gln. These findings are also consistent with a limitation in the transfer of reducing equivalents and a concomitant reduction in the TCA cycle activity in the glia. Interestingly, dietary hyperammonemia doesn't cause any kind of significative alteration in the transfer of reducing equivalents between cytosol and mitochondria.

Conclusion. In summary, we showed that portocaval anastomosis induces important changes in the deuteration pattern of the H3 hydrogens from [2-^{13}C]glu and [2-^{13}C]gln. These results indicate that limitations in the transfer of reducing equivalents between cytosol and mitochondria of neurons and glial cells may underlie the limitations in cerebral TCA cycle flux consistently reported to occur during hepatic encephalopathy.

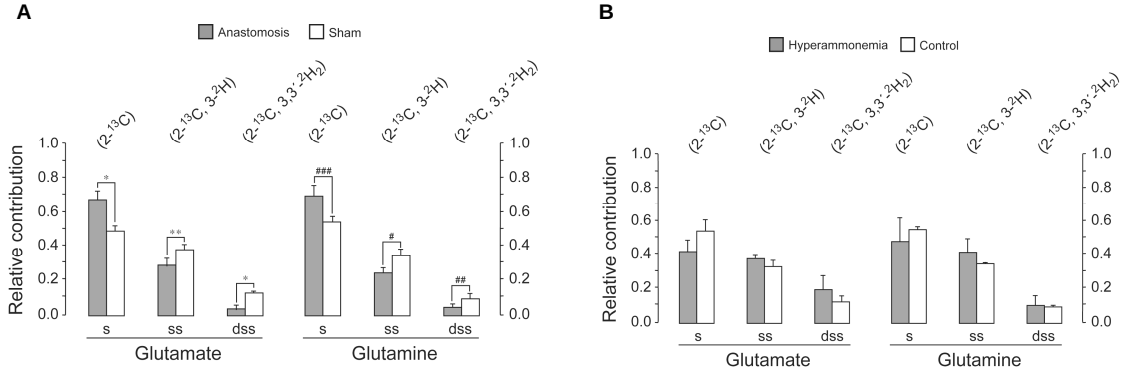

Fig. 1: Relative proportions of [2-^{13}C], [2-^{13}C, 3-^{2}H] and [2-^{13}C, 3,3'-$^{2}H_2$] isotopomers of cerebral glu and gln in sham operated or subjected to portocaval anastomosis (Panel A) and in control or hyperammonemic animals (Panel B). Results are the mean ± S.D. of four extracts in each condition. s: singlet; ss: shifted singlet; dss: doubly shifted singlet. *: P<0.001; **: P<0.02; #: P<0.01; ##: P<0.05; ###: P<0.005.

References. 1. Rodrigues and Cerdán, Concepts in MR. 2005. 27A:1. **2.**Felipo and Butterworth, Prog Neurobiol. 2002. 67:259. **3.**Lee et al., Surgery. 1961. 50:668.