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Effect of Portacaval Anastomosis on Glutamine Synthetase Protein and Gene Expression in Brain, Liver and Skeletal Muscle

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ABSTRACT

The effects of chronic liver insufficiency resulting from end-to-side portacaval anastomosis (PCA) on glutamine synthetase (GS) activities, protein and gene expression were studied in brain, liver and skeletal muscle of male adult rats. Four weeks following PCA, activities of GS in cerebral cortex and cerebellum were reduced by 32% and 37% ($p<0.05$) respectively whereas GS activities in muscle were increased by 52% ($p<0.05$). GS activities in liver were decreased by up to 90% ($p<0.01$), a finding which undoubtedly reflects the loss of GS-rich perivenous hepatocytes following portal-systemic shunting. Immunoblotting techniques revealed no change in GS protein content of brain regions or muscle but a significant loss in liver of PCA rats. GS mRNA determined by semi-quantitative RT-PCR was also significantly decreased in the livers of PCA rats compared to sham-operated controls. These findings demonstrate that PCA results in a loss of GS gene expression in the liver and that brain does not show a compensatory induction of enzyme activity, rendering it particularly sensitive to increases in ammonia in chronic liver failure. The finding of a post-translational increase of GS in muscle following portacaval shunting suggests that, in chronic liver failure, muscle becomes the major organ responsible for the removal of excess blood-borne ammonia.

Keywords: glutamine synthetase; gene expression; portacaval anastomosis; hepatic encephalopathy; liver; skeletal muscle; ammonia

INTRODUCTION

Glutamine synthetase (GS) (EC 6.3.1.2) is responsible for the ATP-dependent amidation of glutamate to glutamine. In liver and in brain, GS is compartmentalized. In the case of liver, GS activities are expressed by a small population of perivenous hepatocytes (Gerbhardt and Mecke, 1983), in brain, GS is localized almost exclusively to astrocytes (Norenberg and Martinez-Hernandez, 1979).

There is a convincing body of evidence to suggest that ammonia toxicity is the major factor in the pathogenesis of hepatic encephalopathy (HE) (Butterworth *et al.,* 1987). Unlike liver, brain and skeletal muscle rely almost exclusively on glutamine formation for the effective removal of excess ammonia (Cooper *et al.,* 1985). In liver failure, it has been suggested that muscle glutamine synthesis becomes an important alternative pathway for removal of blood-borne ammonia via GS (Ganda and Ruderman, 1976).

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In order to further elucidate the molecular basis for the tissue-selective changes in capacity for ammonia removal in chronic liver failure, the present study was undertaken to assess the effects of portacaval anastomosis (PCA) in the rat on GS protein and gene expression in brain, liver and skeletal muscle.

MATERIALS AND METHODS

Animal Surgery

Adult male Sprague-Dawley rats weighing 175-200 g were anesthetized with halothane and an end-to-side PCA was performed essentially according to the procedure of Lee and Fisher (1961). Rats underwent a laparotomy, the inferior vena cava and portal vein were isolated, the inferior vena cava partially clamped (anastomosis clamp, Roboz Instruments Inc., Washington, D.C.), and an elliptical piece of vein, 1.5 times the portal vein diameter was removed. The portal vein was ligated, cut, and an end-to-side anastomosis performed under a dissecting microscope. Total surgery time was <15 min. Sham-operated control rats, matched for weight, were similarly anesthetized, a laparotomy was performed, and the inferior vena cava and portal vein were occluded for 15min.

Following surgery, animals were housed individually under constant conditions of temperature, humidity, and light cycles and were allowed free access to standard laboratory chow (except for sham operated pair-fed rats) and water. The animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals, Vol. 1 (1980) and Vol. 2 (1984) (Canadian Council of Animal Care, CCAC, Ottawa, Canada). Overall mortality for shunted rats was less than 10%.

Tissue Sampling

Groups of rats were sacrificed by decapitation 4 weeks following PCA or sham operation. Brains were rapidly removed on ice and, at the same time, livers and a 1 g sample of vastus lateralis muscle were rapidly removed and immediately frozen. All tissues were stored at -70°C until use.
Blood ammonia was measured in neck blood according to the method of Kun and Kearney (1974)

GS Activities

Glutamine synthetase (EC6.3.1.2) was measured in tissue homogenates using a modification of the procedures described by Patel *et al.* (1983) and Lavoie *et al.* (1987). The assay mixture contained in a final volume of 0.1 ml: imidazole buffer 100 mM, pH 7.2;12.5 mM $MgCl_2$; 20 mM mercaptoethanol; 10 mM ATP; 4 mM ammonium chloride; 13 mM phosphoenolpyruvate; 50 units of pyruvate kinase; 0.16% (v/v) Triton X-100; and 0.6 mg, 0.6 mg and 2.4 mg of brain, liver and skeletal muscle homogenates respectively. Homogenization was made by Teflon pestle in various buffers depending on the tissue: imidazole buffer 100 mM, pH 7.2 for brain; Tris HC1 50 mM-EDTA for liver and skeletal muscle. Following preincubation, 30 min at 37°C, [l-^{14}C]glutamate (specific activity 60 mCi/mmol, New England nuclear) was added to provide a final concentration of 20 mM for brain tissue, 50 mM for liver, and 60 mM for skeletal muscle. Optimal substrate concentrations were derived from a separate series of experiments in tissue homogenates. Boiled homogenates served as blanks in all cases. After incubation at 37°C for 60 min for brain (30 min for liver and muscle) the reaction was stopped by adding 1 ml ice-cold water and the mixture was immediately applied to 3 cm x 0.7 cm columns containing anion exchange resin (Dowex AG 1 x 8, acetate form). Columns were made from 2.5 ml plastic pipet tips (Walter Sarstedt, Princeton, New Jersey) equilibrated by eight successive washings with distilled water. Experiments with standard mixtures of [^{14}C]glutamate and [^{14}C]glutamine showed that, following elution with 5 ml cold (4°C) distilled water, [^{14}C]glutamate was quantitativelyretained on the ion-exchange column. Protein content of homogenates was determined by the methods of Lowry *et al.* (1951). Enzyme activities were expressed as micromoles of glutamine formed per hour per milligram of protein.

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Immunoblotting

Tissues were homogenized in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100,0.5% sodium deoxycholate), and a protease inhibitor mixture consisting of 1mM EDTA, 0.1 uM pepstatin A, 1 ug/ml leupeptin, 1 ug/ml aprotinin, and 100 ug/ml phenylmethyl-sulfonyl fluoride. After centrifugation at 12,000 g for 15 min, protein extracts (50 ug) were boiled for 10 min in loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol; 2% SDS, 0.1 M dithiothreitol, 0.1% bromophenol blue). Proteins were resolved by 8% denaturing SDS-polyacrylamide gels and transferred overnight to nitrocellulose filters. The membranes were blocked for 2 h in Tris-buffered saline (TBS) containing 5% dry milk and 0.1% Tween 20, then incubated for 1 h with a mouse monoclonal antibody directed against rat GS (Transduction laboratories, Lexington, KY). The blots were subsequently probed with anti-mouse horseradish peroxidase-conjugated antiserum (Promega, Madison, Wl) diluted 1/40,000 in the same buffer. After extensive washing with TBS, the peroxidase activity was detected by cheminulescence using the ECL detection system (Amersham, Arlington Heights, IL).

RNA Extraction

Total RNA was extracted using TRI Reagent (MRC Inc., Ohio) according to the manufacturer's protocol. Putative contaminating DNA was eliminated by adding 100 U of RNase-free DNase I per 50u g of total RNA at 37°C for 1 h. Purified RNA was then extracted with phenol, precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. RNA samples were kept at -70°C until use.

RT-PCR Analysis

Glutamine synthetase expression was investigated by the reverse transcription-polymerase chain reaction (RT-PCR) B-Actin was used as an internal standard to monitor loading variations. Total RNA (0.5 ug) was mixed with 10 mM Tris-HCl_2 (pH 8.3), 1.0 mM $MgCl_2$, 50 mM KC1, 0.01%(w/v) bovine serum albumin, 100 uM dNTPs, primers at 1 uM each, AMV reverse transcriptase (80 U/ml), Taq DNA polymerase (20 U/ml) and 50 uCi/ml [a32P]dCTP (3000 Ci/mmol), for a total reaction volume of 50 ul. The reactions were initially heated at 50°C for 15 min followed by PCR at 95°C for 30 sec, 59°C for 45 sec and 72 C for 1 min. Amplification efficiency conditions were determined after a kinetic study, to ensure all experiments were performed within the exponential phase of amplification where PCR product remains proportional to initial template concentration (data not shown). B-Actin and GS mRNAs were amplified for 24 cycles. After amplification, the samples were electrophoresed onto 8% polyacrylamide gels, dried, autoradiographed at -70°C with an intensifying screen. Each band was excised and Cerenkov radiation was quantitated using a B-counter. Oligonucleotide primers were designed using the PRIME program (Genetic Computer Group, Wisconsin) and synthesized by the Sheldon Biotechnology Center (McGill University, Quebec) based on the following GeneBank accession numbers: X00351 (B-Actin, Ponte et al, 1984), and M91652 (GS, Mill et al, 1991). The forward and reverse primer sequences were as follows: CATCCCCCAAAGTTCTAC and CCAAAGCCTTCATACATC (B-Actin, 347bp); ACCTGACAAATGGCCCTAC and ACCAAAAAATAACCCCCC (GS, 482bp). The specificity of the oligonucleotide primers was verified using the program BLASTN (National Center for Biotechnology Information, Bethesda, MD).

Statistical Analyzes

Between-group comparisons were made using unpaired Student t-test with Bonferroni correction. A p value of <0.05 was considered to indicate a significant difference.

RESULTS

Effects of PCA on GS activities in liver, muscle and brain regions are shown in Table 1.

Table 1. Effects of portacaval anastomosis on GS activities in liver, brain and skeletal muscle.

		GS Activity (umole/mg protein/h)
Tissue	Sham-operated	PCA
Liver	1.08±0.08	0.36±0.2**
Muscle	0.29±0.03	0.45±0.05*
Brain		
Cerebral Cortex	2.01 ±0.15	1.32±0.10*
Cerebellum	3.90±0.41	2.81±0.26*

Values represent mean ± S.E. of duplicate determinations from 6 rats per treatment group. Values significantly different from sham-operated control indicated by *p<0.05, **p<0.01 by unpaired Student t-test.

GS activities in tissues from sham-operated control rats were in general agreement with previously-published studies (Girard and Butterworth, 1992; Girard *et al,* 1993). PCA resulted in a 67% decrease (p<0.01) in GS activities in liver and a significant 52% increase (p<0.05) of enzyme activity in skeletal muscle. A significant loss of GS activity was observed in both cerebral cortex and cerebellum of PCA rats.

Effects of PCA on GS protein in cerebral cortex, cerebellum, muscle and liver are shown in Figure 1 and Table 2. As can be seen, there was no significant effect of PCA on GS protein in brain regions or in muscle; in the case of muscle, both sham-operated and PCA rat samples showed a wide variation in GS protein. In the case of liver, GS protein was severely decreased in PCA rats compared to sham-operated controls (Figure 1); in one case (lane 11) GS protein following shunting was below the level of detection.

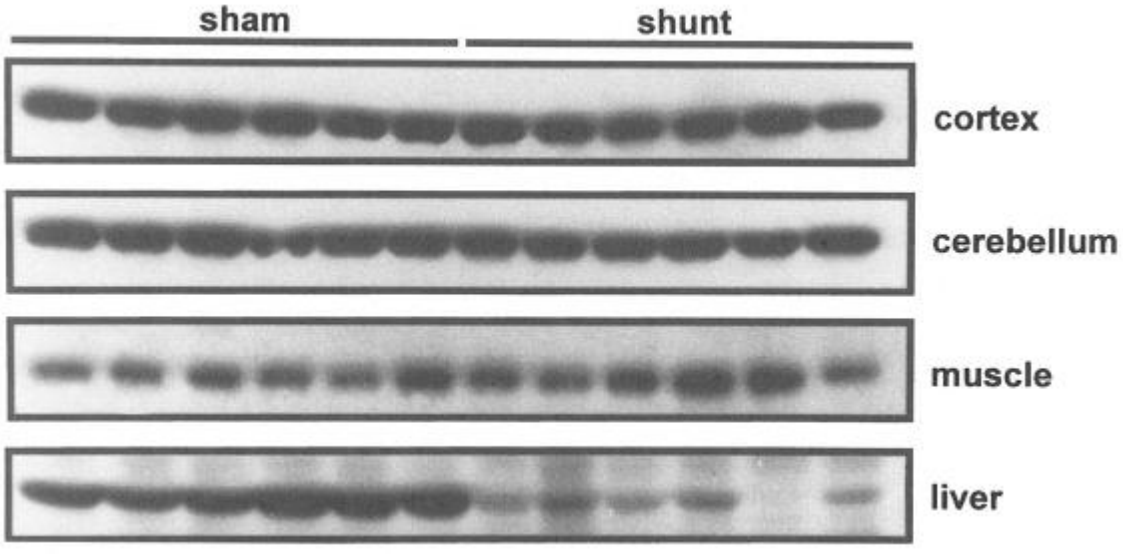

Figure 1: Effect of portacaval anastomosis (shunt) on GS protein in brain regions, muscle and liver compared to sham-operated controls (sham). Note the variability in GS protein in muscle in both experimental groups and the severe loss of GS in liver of shunted animals consistent with a loss of GS-rich perivenous hepatocytes.

Table 2. Effect of portacaval anastomosis (shunt) on GS protein in liver, brain and skeletal muscle.

	GS Protein (O.D. units)	
Tissue	Sham-operated	Shunt
Liver	0.41±0.01	0.06±0.02*
Muscle	0.35±0.02	0.36±0.02
Brain		
Cerebral cortex	0.60±0.04	0.58±0.05
Cerebellum	0.54±0.01	0.51 ±0.19

Values represent mean ± S.E. of duplicate determinations from 6 rats per treatment group. Values significantly different from sham-operated controls indicated by *p<0.05 by unpaired Students t-test.

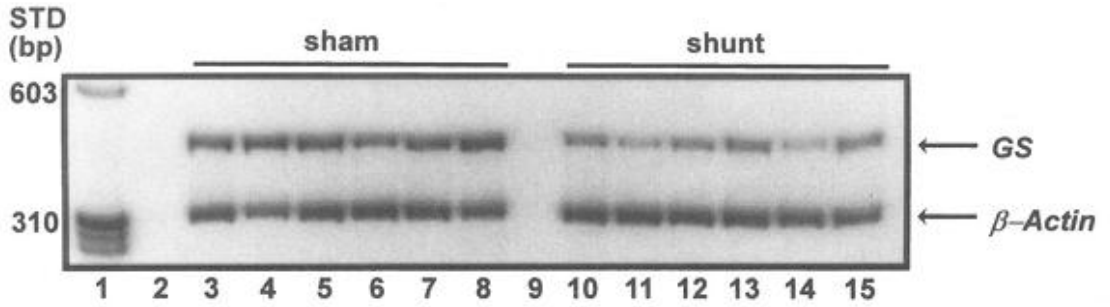

Figure 2: Decreased glutamine synthetase (GS) gene expression in the liver of rats following portacaval anastomosis. Total RNA was extracted from the liver of animals following portacaval anastomosis (shunt, lanes 3-8) or sham operation (sham, lanes 10-15). B-Actin (347 bp) and glutamine synthetase (482 bp) were reverse-transcribed and amplified by PCR for 24 cycles. Lane 1: molecular weight standards (bp); lanes 2 and 8: AMV reverse transcriptase was omitted (as a negative control) from the reaction mixture.

In Figure 2, the effects of PCA on GS mRNA in liver are presented. As was the case with GS protein, GS mRNA in liver of PCA rats is severely reduced compared to B-actin (reporter gene). Percent reduction in expression ranged from 50 to 80% compared to sham-operated control animals.

DISCUSSION

Results of the present study reveal tissue-selective alterations of GS expression following portacaval shunting. In the case of liver, GS activities were reduced to one third of control values, confirming a previous report (Girard and Butterworth, 1992). Reduced liver GS activities undoubtedly result from a selective loss of perivenous hepatocytes following PCA. Immunohistochemical studies reveal that GS is preferentially localized in perivenous area of the liver lobule (Gerbhardt and Mecke, 1983); perivenous GS removes ammonia which escapes periportal urea synthesis. It has been reported that GS activity is reduced by 80% in human cirrhotic liver compared to controls (Kaiser *et al.,* 1988) leading to the proposal that the pathogenesis of hyperammonemia in chronic liver disease involves the impairment of the perivenous ammonia-scavenging GS-rich hepatocytes. CCl_4-induced liver injury also results in loss of perivenous hepatocytes and a concomitant loss of GS activity. Results of the present study reveal that PCA, in the absence of parenchymal liver cell necrosis, has a similar effect, namely the loss of GS activity as well as GS protein and gene expression in the liver. GS is the principal mechanism responsible for ammonia removal by brain under both normal

and hyperammonemic conditions (Cooper *et al.,* 1985). GS is localized predominantly in astrocytes (Norenberg and Martinez-Hernandez, 1979). Consequently, neurons do not have an effective defense mechanism against increases of blood-borne ammonia and must rely on the neighbouring astrocyte for ammonia removal. The vulnerability of neurons to increased ammonia is exacerbated by the findings of the present study that GS is not induced in brain in chronic hyperammonemia; rather there appears to be a significant loss of GS activity in cerebral cortex and cerebellum of portacaval-shunted rats. Similar findings of a lack of induction of GS in brain in hyperammonemic conditions including experimental and human liver failure were previously reported (Cooper *et al.,* 1985; Lavoie *et al.,* 1987; Giguere *et al.,* 1989; Butterworth *et al.,* 1988; Qureshi *et al.,* 1995; Girard *et al.,* 1993). This lack of induction of brain GS in conditions of chronic liver failure is responsible for the precipitously high levels of brain ammonia which may occur in experimental animals with chronic liver failure (Butterworth *et al.,* 1988).

In addition to resulting in seriously compromised capacity for ammonia removal, a modest loss of GS activity in brain following PCA could have serious consequences for glutamatergic synaptic regulation (Butterworth, 1992). Termination of the action of synaptically-released glutamate relies almost exclusively on high affinity uptake into the perineuronal astrocyte where it is transformed into glutamine via the action of GS. It is interesting therefore that, in studies using astrocytes cultured from different brain regions in the rat, high affinity glutamate uptake capacity was found to be highly correlated with regional GS activities (Hansson, 1986). Reductions in brain GS activity, as observed in the present and a previous study (Girard *et al.,* 1993), would therefore be expected to result in decreased astrocytic glutamate uptake following PCA. In favour of this possibility, several reports describe increases of extracellular brain concentrations of glutamate in the brains of PCA rats (Moroni *et al.,* 1983; Tossman *et al.,* 1987), a finding which is consistent with diminished astrocytic uptake in the brains of these animals.

In the case of skeletal muscle, GS activities were significantly increased following portacaval shunting, a finding which confirms a previous report (Girard and Butterworth, 1992). GS protein expression showed a wide variability in both sham-operated and PCA groups of animals, a finding which may reflect a variable loss of muscle mass in chronic liver failure as a result of nutritional factors. Further studies are necessary in order to address this issue. It has been suggested that muscle ammonia uptake is increased in chronic liver failure and that the subsequent increase in glutamine synthesis capacity is a major alternative pathway for ammonia detoxification (Lockwood *et al.,* 1979; Girard and Butterworth, 1992). Results of the present study confirm this possibility. Furthermore, results of the present study suggest that the increased GS activity in skeletal muscle in chronic liver failure is not due to induction of GS gene expression in muscle. Rather, the finding of unaltered GS protein and mRNA in muscle following PCA suggests that the increased GS activities observed are the result of a post-translational modification of the enzyme.

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