Published in final edited form as:
Metab Brain Dis. 2014 December ; 29(4): 1041–1052. doi:10.1007/s11011-013-9442-y.

Reduced clearance of proteins labeled with diisopropylfluorophosphate in portacaval-shunted rats

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Abstract

Portacaval shunting is a model for hepatic encephalopathy that causes chronic hyperammonemia, disruption of metabolic, signaling, and neurotransmitter systems, and progressive morphological changes. Exposure of cultured cells to ammonia raises intralysosomal pH and inhibits proteolysis, and the present study tested the hypothesis that proteolytic capacity is diminished in portacaval-shunted rats. Proteins were labeled in vivo with tracer doses of diisopropylfluorophosphate (DFP) and clearance of label was assayed. This approach labeled proteins independent of protein synthesis, which is reported to be altered in shunted rats, and avoided complications arising from re-utilization of labeled amino acids that causes underestimation of degradation rate. Characterization of DFP labeling showed that protein labeling was fast, about 50% of the label was released during a 24h interval, labeling by DFP metabolites was negligible, inhibition of brain acetylcholinesterase was not detectable, and labeling by [^{3}H]- and [^{14}C]DFP was equivalent. To assay degradative capacity, proteins were first labeled with [^{3}H]DFP, followed by labeling with [^{14}C]DFP that was given 24 or 72h later. The ^{3}H/^{14}C ratio in each animal was used as a relative measure of removal of ^{3}H-labeled proteins. ^{3}H/^{14}C ratios were generally significantly higher in portacaval-shunted rats than in controls, consistent with reduced proteolytic capacity. Assays of amino acid incorporation into brain protein generally replicated literature reports, supporting the conclusion that protein synthesis unlikely to be markedly inhibited and amino acid recycling influences calculated protein synthesis rates in shunted rats. Therapeutic strategies to reduce ammonia level would help normalize lysosomal functions and protein and lipid turnover.

Keywords

ammonia; brain; liver; portacaval shunt; protein synthesis; proteolytic capacity

INTRODUCTION

Ammonia is a neurotoxin that disrupts many metabolic, transport, energetic, signaling, neurotransmitter, and blood-brain barrier systems, causing complex, deleterious effects on brain function (Cooper 1990; Cooper and Plum 1987; Duffy and Plum 1982; Albrecht et al. 2010; Butterworth 2011; Hazell and Butterworth 1999; Palomero-Gallagher and Zilles 2013;

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The authors declare no conflict of interest.

Skowronska and Albrecht 2013, 2012; Llansola et al. 2013). Portacaval shunting is a model for liver disease that chronically elevates the level of ammonia in blood, brain and other body tissues by diverting blood from the portal vein into the general circulation (Williams et al. 1972). Astrocytes are the primary site for brain ammonia detoxification, and the action of glutamine synthetase quickly incorporates ammonia into glutamine, increasing glutamine levels in shunted rats (Cooper 2011, 2012). Astrocytes exhibit progressive morphological changes after construction of the shunt: during the first few weeks astrocytes swell, membrane-bound cytoplasmic vacuoles appear, and glycogen granules disappear; after 4 weeks, swelling subsides, vacuoles disappear, and there are increases in endoplasmic reticulum, glycogen granules, ribosomes, and mitochondria; between 8–12 weeks, there are further increases in the endoplasmic reticulum, mitochondria, and lysosomes, accumulation of filaments, and appearance of dense granular bodies that resemble lipofuscin granules, fat inclusions, and lipid droplets; at later times there are degenerative changes; and structural changes also occur in cerebral vessels, oligodendroglia, and neurons (Zamora et al. 1973; Norenberg 1977; Norenberg and Lapham 1974; Laursen 1982). Concentration- and time-dependent effects of ammonia on morphology of cultured astrocytes have been documented (Gregorios et al. 1985a; Gregorios et al. 1985b).

Abnormal protein turnover arising from disruption of amino acid transport, pool sizes, and metabolism may be one factor underlying hyperammonemia-evoked morphological changes. For example, portacaval shunting alters the blood-brain barrier amino acid transport and brain levels of many amino acids (Jeppsson et al. 1983; Jeppsson et al. 1979; Mans et al. 1984; James et al. 1978). Discordant results have been obtained for in vivo rates of incorporation of various labeled amino acids into brain protein of adult portacaval-shunted rats, with reports of decreases (Wasterlain et al. 1978; Lundborg and Hamberger 1977; Helewski and Konecki 1994) or no change (Dunlop et al. 1984; Cremer et al. 1977). Acute exposure to ammonia also inhibits label incorporation into protein in slices from immature rat brain (Schott et al. 1984). Ammonia and other weak bases are known to accumulate in lysosomes of cultured cells, thereby raising intralysosomal pH and inhibiting lysosomal enzymes involved in proteolysis and lipid degradation, causing drug-induced lysosomal storage diseases (Seglen 1983; Lüllmann-Rauch 1979). Treatment of cultured cells and animals with lysosomotropic agents is associated with lysosomal swelling, greater numbers of autophagosomes, and accumulation of phospholipids and gangliosides in vitro and in vivo (Seglen 1983; Kovacs et al. 1982; Nilsson et al. 1981; Lüllmann-Rauch 1979). Thus, protein degradation, as well as synthesis, may be compromised by chronic elevation of ammonia levels. To our knowledge, proteolysis has never been examined in brain of portacaval-shunted rats.

Protein degradation is commonly assayed by determination of rates of loss of label from protein pre-labeled with ^{3}H- or ^{14}C-labeled amino acids. However, recycling of labeled amino acids derived from proteolysis back into brain protein causes large underestimates of protein degradation rates unless special precautions are taken to minimize label reutilization (Dunlop 1983). For example, the half life of total brain protein is 3.3–8.7 days when labeled with [^{14}C] bicarbonate, a precursor that minimizes recycling (Chee and Dahl 1977), compared with 8.4–16.5 days when labeled with [1-^{14}C] leucine (Chee and Dahl 1978). Alternative procedures using various reagents that do not depend on protein synthesis have

also been used to evaluate protein catabolism. For example, N-ethylmaleimide (NEM) mainly labels cysteinyl residues, and it has been used to determine half lives of NEM-labeled muscle protein in vivo, and demonstrate label accumulation in lysosomes that is enhanced by chloroquine, a weak base that inhibits proteolysis (Gerard et al. 1988; Gerard et al. 1977; Gerard and Schneider 1979). Suicide inhibitors are highly-selective reagents that are covalently bound to functional groups at the active site of specific enzymes and cause loss of enzymatic activity (Alston 1981; Rando 1984). Low concentrations of diisopropylfluorophosphate (DFP) and other organic phosphates specifically inhibit many serine hydrolases (esterases and proteases, e.g., acetylcholinesterase, ali-esterase, chymotrypsin, and trypsin) by binding at their active sites (Cohen et al. 1959; Jansz et al. 1959), and these reagents have recently been found to also label tyrosine moieties in other proteins (Schopfer et al. 2010; Casida and Quistad 2005). Many studies have used labeled DFP and its analogs to evaluate aspects of esterase biology, such as tissue distribution of cholinesterase (Jandorf and McNamara 1950), turnover of blood plasma, red cells, and platelets (Bithell et al. 1967; Cohen and Warringa 1954), localization and quantification of acetylcholinesterase in motor endplates of muscle (Rogers et al. 1969; Salpeter 1967), turnover of rat brain acetylcholinesterase (Goossens et al. 1984), and mapping of brain acetylcholinesterase (Irie et al. 1993). Large doses of unlabeled DFP have been used to study reappearance of acetylcholinesterase activity (Austin and James 1970).

In the present study, we tested the hypothesis that ammonia reduces the capacity for lysosomal protein degradation in portacaval-shunted rats by measuring clearance of label from DFP-labeled protein; this approach avoids complications arising from amino acid recycling and changes in protein synthesis. Because brain ammonia levels vary among portacaval shunt models and the portacaval shunt-protein synthesis literature is discordant, incorporation of labeled amino acids into brain protein was first determined to further characterize our rat model that produces consistent levels of ammonia over many years (Cruz and Dienel 1994; Cruz and Duffy 1983; Ehrlich et al. 1980; Gjedde et al. 1978; Lockwood and Duffy 1977; Hindfelt et al. 1977; Gjedde et al. 1976; Cooper et al. 1985). Then, clearance of label from DFP-labeled protein was evaluated in tissues of portacaval-shunted and control rats. Label clearance rates are considered to represent lysosomal degradative capacity, not the normal degradation rates that would include contributions of other proteases and of proteosomes.

METHODS

Animals and operative procedures

Male Wistar rats were purchased from Charles River Breeding Farms (Wilmington, MA) and had end-to-end portacaval anastomoses constructed in our laboratory by the suture technique as previously described (Cruz and Duffy 1983). Rats were used at intervals after construction of the portacaval shunt (PCS), and sham-operated rats were used as controls. All animal use procedures were in strict accordance with the *NIH Guide for Care and Use of Laboratory Animals*, and were reviewed and approved by the local animal care and use committee.

Protein synthesis assays

Incorporation of label from [1-^{14}C]valine, L-[4,5-^{3}H(N)]lysine, and L-[alanine-1-^{14}C]phenylalanine into protein was determined using the precursor pool flooding procedure of Dunlop et al. (1975), as previously described (Dienel et al. 1980). This method uses a large (i.e., flooding) dose of the precursor amino acid with the goal of equilibrating the specific activity of the aminoacyl-tRNA with that of the amino acid pools in blood and tissue. The procedure reduces effects of re-utilization of amino acids derived from proteolysis that would cause underestimation of calculated rates of labeled amino acid incorporation into protein, and it should also reduce effects on pool size arising from changes in amino acid transport or metabolism that may occur during pathophysiological states (see Discussion). In brief, awake rats were injected intraperitoneally with [^{14}C]valine (7.5 mmol/kg, 2.66 μCi/mmol), [^{3}H]lysine (10 mmol/kg, 50 μCi/mmol), or [^{14}C]phenylalanine (5 mmol/kg, 8.27 μCi/mmol), and killed one hour later. The brains, livers, and kidneys were quickly removed; brain tissue assays were carried out using whole brain or dissected cerebral cortex. Tissue samples were weighed and homogenized in 4 volumes of ice-cold 5% trichloroacetic acid (TCA). After sitting on ice for 30 min, the samples were centrifuged at 10,000g for 20 min, and portions of the supernatants were assayed for their ^{3}H or ^{14}C contents by liquid scintillation counting with external standardization; the quantity of acid-soluble ^{3}H or ^{14}C reflects the 'flooded' tissue amino acid pool that reduces effects of reutilization of unlabeled amino acids on rates of incorporation of labeled amino acids into protein. Metabolism of the 1-^{14}C-labeled amino acids produces $^{14}CO_2$ that would be released during the acid precipitation step, whereas $^{3}H_2O$ arising from metabolism of [^{3}H]lysine would be contained in the acid-soluble fraction. Separate portions of the [^{3}H]lysine-containing TCA supernatants were, therefore, dried to quantify volatile and non-volatile components. The acid-insoluble fractions were sequentially washed (re-suspended and centrifuged) to remove lipids and nucleic acids as follows: twice with ice-cold 5% TCA, once with hot 5% TCA (90oC for 30 min), three times with $CHCl_3$:CH_3OH (1:1 by vol.), and diethyl ether. The protein pellets were dried, weighed, wetted with 50% methanol, dissolved in Protosol™ (DuPont-NEN), and assayed for their ^{3}H or ^{14}C contents.

Protein labeling with DFP

Unlabeled DFP was obtained from Aldrich Chemical Co and diluted with anhydrous propylene glycol. [1,3-^{3}H]DFP (6.5 Ci/mmol) and [1,3-^{14}C]DFP (103 mCi/mmol) were obtained from Amersham and DuPont NEN, respectively, and diluted with anhydrous propylene glycol to about 1.0 or 0.1 mCi/ml, respectively. The day before the experiment, rats were deeply anesthetized, and a PE-50 catheter was inserted into a jugular vein and exteriorized to the back; the next day, awake rats were given intravenous injections of [^{3}H]DFP (300 μCi/kg) or [^{14}C]DFP (15 or 30 μCi/kg, as indicated in the text or tables). At timed intervals after the injection, rats were deeply anesthetized, perfused with 0.15M NaCl via the ascending aorta to remove blood from the tissues prior to determination of the ^{3}H or ^{14}C contents of tissue protein (see below). In subsequent experiments, [^{3}H]DFP was injected on day one, followed by [^{14}C]DFP on day 2 or day 3, as described in the text. The rational for use of double-label injections was that clearance of ^{3}H from protein could be evaluated in individual animals by normalizing the ^{3}H remaining in protein to labeling by

[^{14}C]DFP that would serve as an 'internal reference standard'. ^{3}H/^{14}C ratios were compared in groups of control and portacaval-shunted rats, thereby greatly reducing the number of operated animals required for the study.

After perfusion to remove blood, brain, liver, and kidney were rapidly removed and weighed. When whole brain, liver, and kidney were assayed, tissues were homogenized in four volumes of ice-cold 5% TCA, and the TCA-insoluble protein pellets were purified as described above. When subcellular fractions of brain were prepared, brains were homogenized in four volumes of ice-cold 0.32M sucrose and fractionated by differential centrifugation according to modifications of the procedure of Gray and Whittaker (1962). P_1 + P_2, the particulate fraction comprised of cell debris, nuclei, mitochondria, synaptosomes, and myelin, was obtained by centrifugation at 17,000g for 20 min. The resulting supernatant fraction, S_2, was fractionated by centrifugation at 100,000g for 60 min to produce P_3, the microsomal particulate fraction, and S_3, the cell-soluble fraction. Samples of homogenate and subcellular fractions were assayed for acetylcholinesterase activity (Ellman et al. 1961), and their ^{3}H and ^{14}C contents were determined in separate portions of each sample that were added to 5 volumes of ice-cold 5% TCA and processed as described above to precipitate protein and remove lipid and nucleic acids. In some experiments in which only the ^{3}H/^{14}C ratios were determined, portions of the homogenates were applied to filter discs, the discs were placed in ice-cold 5% TCA, batch-washed as described above (Mans and Novelli 1961), the discs were wetted and dissolved in Protosol, then assayed for ^{3}H and ^{14}C contents. Preliminary experiments (not shown) yielded similar ^{3}H/^{14}C ratios for ten paired samples assayed by the disc method and the washed pellet method.

Statistics

Comparisons between means of two group were made with the unpaired t-test. Multiple comparisons of independent groups against the same control group were made with analysis of variance (ANOVA) followed by Dunnett's test. $p<0.05$ was considered significant.

RESULTS

Uptake of flooding doses of labeled amino acids and incorporation into protein

Rats transiently lose weight after construction of the portacaval shunt, but by 4–8 weeks after the surgical procedure the body weights of shunted and sham-operated control rats were similar (Tables 1 and 2, (Cruz and Duffy 1983)). At one hour after injection of a flooding dose of [^{3}H]lysine, the net accumulation of ^{3}H in the brain non-volatile TCA-soluble fraction (assumed to be mainly [^{3}H]lysine) was similar in control and 8-week shunted rats (Table 1). Based on the specific activity of the injectant, the brain lysine concentration was estimated to be 0.9 and 0.77 μmol/g, respectively, which is 2.8- and 2.4-fold higher than the normal brain lysine content of 0.32 μmol/g (Mans et al. 1984). The estimated lysine concentration in liver of shunted rats was more than twice that in control rats, i.e., 36 vs. 15 μmol/g, and was much higher than in brain (Table 1), probably due to restricted lysine transport across the blood-brain barrier.

Incorporation of [^{3}H]lysine into brain protein was reduced by about 15%, whereas that into liver protein was depressed by 57% (Table 1). Because (i) the high dose of injected [^{3}H]lysine produced a relatively modest increase in brain lysine level in both control and shunted rats, and (ii) interpretation of the 50% decrease of [^{3}H]lysine incorporation into brain protein in 8-week shunted rats in a previous study (Wasterlain et al. 1978) was questioned on the basis of relatively small increases in the acid-soluble precursor pool that may not result in equilibration of the [^{3}H]lysyl-tRNA specific activity with those of brain and plasma [^{3}H]lysine pools (Dunlop et al. 1984), label incorporation into protein was also assayed with phenylalanine and valine.

Flooding with [^{14}C]phenylalanine nearly doubled the size of the acid-soluble labeled precursor pool in brain and liver of the 8-week shunted compared with control rats (Table 1). The phenylalanine concentrations in brain were estimated to be about 0.34 and 0.63 μmol/g in control and shunted rats, respectively. These values correspond to about 7.7 and 14.3 times the normal brain phenylalanine level of 0.044 μmol/g (Mans et al. 1984), indicating that phenylalanine flooding caused larger relative increases in pool size than lysine. Incorporation of [^{14}C]phenylalanine into brain protein in 8-week shunted rats was reduced by about 22%, whereas that into liver protein tended to be increased but not reach statistical significance (Table 1).

Flooding with [^{14}C]valine increased the brain valine pool by 1.5-2-fold above control during the 3 day to 12 week interval after portacaval shunting, whereas the liver pool was statistically significantly increased only at 12 weeks, and it was unchanged in kidney (Table 2). The concentration of brain valine calculated from the injectant specific activity ranged from about 1.4 to 2.8 μmol/g, which is similar to that reported by Dunlop et al. (1984). These levels were 19 and 39 times the normal brain valine pool size, respectively (0.072 μmol/g; (Mans et al. 1984)). Thus, valine flooding increased the relative size of the brain acid-soluble pool more than phenylalanine or lysine.

Incorporation of [^{14}C]valine into whole-brain protein was not altered between 3 days and 12 weeks after portacaval shunting (Table 2), consistent with results in 4-week shunted rats obtained with [^{14}C]valine flooding (Dunlop et al. 1984). On the other hand, incorporation of [^{14}C]valine into liver protein was increased 1.4–2.2-fold at all times after shunting, and that into kidney protein was elevated by about 35% at 3 days and 2 weeks (Table 2).

To summarize, impairment of amino acid incorporation into brain protein was not detected when flooding of the acid soluble pool was greatest (i.e., with valine), whereas 15–20% decrements were observed when the flooding doses of amino acids produced smaller incremental increases in the brain acid-soluble pool size (Tables 1 and 2). Amino acid incorporation into liver protein of shunted rats was increased for the two neutral amino acids, contrasting the 50% decrement with lysine. The cause(s) for this apparent discrepancy remain to be established, but may be related to the effectiveness of pool flooding and extent of equilibration of the aminoacyl-tRNA in tissue with that in the blood and tissue amino acid pools (see Discussion).

Labeling brain protein with DFP and label clearance

Initial experiments characterized protein labeling by [^{3}H]DFP and rate of release of label from brain protein to determine if the procedure could provide a reasonably sensitive measure of proteolytic capacity. Labeling of the acid-insoluble fraction of whole brain was similar in individual rats killed at 0.5, 1, 2, and 4h, indicating that maximal labeling occurs within 30 min after pulse intravenous injection of the tracer. About 0.25% of the injected dose of [^{3}H]DFP was protein bound in brain at 4h. Autoradiographic analysis of ^{14}C-DFP labeled brain revealed a relatively uniform distribution throughout the brain parenchyma with greater labeling of gray compared with white matter and much higher labeling of the meninges and choroid plexus (data not shown). When groups of rats were killed at 4, 24, and 48h after pulse labeling with [^{3}H]DFP, about 60% of the label was released during the first 24h interval, another 35% during the second 24h interval (Table 3), and a plot of log(dpm/g) vs. time yielded a straight line ($r^2 = 0.998$). Subcellular fractionation studies revealed similar rates of label clearance during the first 24h in whole homogenate and three major subcellular fractions, $P_1 + P_2$, P_3, and S_3. The distribution of protein-bound [^{3}H]DFP was 35, 4, and 61% in the $P_1 + P_2$, P_3, and S_3 fractions, respectively, contrasting the distribution of acetylcholinesterase activity in these fractions, i.e., 80, 9, and 11%, respectively. Thus, most of the [^{3}H]DFP is primarily bound to proteins other than acetylcholinesterase. Tracer doses of [^{3}H]DFP did not measurably inhibit brain acetylcholinesterase activity, and functional activity of cholinergic pathways are unlikely to be affected until the dose of DFP exceeds 0.1 mg/kg (Table 4). Sequential washes of the initial acid-insoluble pellet from the $P_1 + P_2$ fraction with hot TCA and $CHCl_3$:CH_3OH (see Methods) released only about 12% (n = 7) of total wash counts, indicating that nucleic and lipids are poorly labeled by DFP, supporting the conclusion that proteins are the major targets of DFP. Variable percentages of the TCA-soluble fraction in the subcellular fractions were volatile (data not shown), suggesting the presence of ^{3}H-metabolites in the tissue.

To determine if label reutilization may interfere with the label clearance assays, labeled metabolites of DFP were tested for incorporation into protein. The DFP was first inactivated by incubation in 0.1N NaOH to hydrolyze the P-F bond and produce diisopropylphosphate (DIP) that was neutralized and injected intravenously into two rats each for ^{3}H- and ^{14}C-labeled DIP. At 48h after pulse labeling, the percent of brain protein labeling by [^{3}H]DIP was only 2.7 and 3.7% of that of [^{3}H]DFP, and labeling by [^{14}C]DIP was only 1.3 and 1.7% that of [^{14}C]DFP. Thus, incorporation of ^{3}H or ^{14}C arising from metabolism of DIP in various body organs into newly-synthesized or existing brain protein is small, and label recycling is minimal.

Equivalence of ^{3}H- and ^{14}C-labeled DFP for protein labeling was evaluated by injecting a mixture of the two tracers and determining ^{3}H/^{14}C ratios in purified protein. The ratios were similar at 4h (14.2 ± 0.9, mean ± SD, n = 3) and 48 h (14.1, 13.7, n=2). Constancy of the ratios with time supports the conclusion that clearance of both labeled tracers is the same so that the one tracer can be used for assessment of label clearance relative to the subsequent labeling by the second tracer injected into the same animal at a specified interval after the first tracer.

Different experimental intervals between the pulse injections of [^{3}H]DFP and [^{14}C]DFP were tested to evaluate label release in shunted rats compared with parallel groups of controls (Table 5). In the experiment 1, the interval between the two tracers was 24h, and rats were killed 24h after the second injection. The ^{3}H/^{14}C ratios were significantly higher in the whole homogenate and $P_1 + P_2$ fractions of brain isolated from 4–8 week shunted rats and in their liver homogenates compared with controls; the ratios were also higher but did not reach statistical significance in the brain S_2 fraction and kidney homogenate (Table 5). In experiments 2–4, the [^{14}C]DFP was injected 72h after the [^{3}H]DFP and the rats were killed one hour later. In experiment 2, all tissues from the 8–9 week shunted rats had greater label retention compared with controls (Table 5). In addition to the organs shown in Table 5, the ^{3}H/^{14}C ratios were also determined in pancreas, and the ratios were also higher in 8-week shunted rats (8.6 ± 0.9) compared with controls (5.6 ± 0.8, $p < 0.05$). Thus, the two different label clearance time intervals in experiments 1 and 2 gave similar results, slower clearance in portacaval shunted rats.

Experiments 3 and 4 evaluated effects of duration of portacaval shunting on label retention. During the first week after shunting, the ^{3}H/^{14}C ratios in shunted rats were significantly greater than control, indicating a rapid response to shunting. The ratios in experiment 3 were, however, much lower than the other experiments using the 72h interval between injections of labeled DFP. This was due in part, to the higher dose of [^{14}C]DFP used in experiment 3 (i.e., 30 μCi/kg vs. 15 μCi/kg for experiments 2 and 4) and, perhaps, also due to use of ether anesthesia during the injections to minimize stressing the rats with newly-constructed portacaval shunts (see Table 5, legend). At 2–12 weeks after construction of the portacaval shunt, only a few selected samples in different tissues had statistically-significantly greater label retention compared with controls (Table 5, experiment 4).

To summarize, the ^{3}H/^{14}C ratios in experiments 1–4 were variable, but expression of the ratios as percent of respective controls for each experiment illustrates the tendency for higher ratios in most samples from the shunted rats (Table 5), consistent with slower release of ^{3}H from the DFP-labeled protein. Similar effects in subcellular fractions of brain and different tissues support the notion that a common mechanism, i.e., increased intralysosomal pH due to hyperammonemia, may cause widespread impairment of lysosomal function throughout the body after construction of the portacaval shunt.

The specific cause(s) for ratio variability were not identified, but different batches of labeled DFP were used for each experiment and could affect the resulting ratios. Also, if the amount of target protein changed with time after shunting, e.g., in response to diminished lysosomal function due to ammonia-induced increases in pH, the amount of labeling by DFP would be affected. Acetylcholinesterase is one target for labeling by DFP, and its brain activity was similar in 8–9 week shunted and control rats when assayed in vitro under optimal conditions such that enzyme activity reflects enzyme amount (Table 4). However, binding of [^{14}C]DFP to protein (dpm/mg protein dry weight) at 1h after pulse labeling was higher in tissues from 8–9-week shunted rats compared with the respective control tissues (samples from experiment 2, Table 5; n=5/group): brain subcellular fractions, $P_1 + P_2$ (62.1 ± 14.5 vs. 34.7 ± 7.1, $p = 0.064$), S_2 (209.6 ± 47.4 vs. 112.7 ± 20.8, $p = 0.049$); liver (30,638 ± 4,709 vs. 8,209 ± 2,065, $p = 0.0012$), kidney (158,174 ± 21,222 vs. 62,705 ± 13,235, $p = 0.0026$), and

pancreas (14,983 ± 1,733 vs. 10,780 ± 1,504, p = 0.052). Increased labeling by DFP suggests that target protein levels are upregulated in the shunted rats, but the identity of the protein(s) that increase in amount and the details of the time course(s) were not determined in the present study. However, as long as target protein amounts are stable over the 3-day experimental interval, the ratio of [^{3}H]DFP relative to [^{14}C]DFP in the same animal should account for differences in the magnitude of labeling in different experimental groups.

Another factor that could influence label loss and ^{3}H/^{14}C ratios in experiments with different intervals between injection of [^{3}H]DFP and [^{14}C]DFP is de-alkylation of enzyme-bound diisopropylphosphate (DIP) to form monoisopropylphosphate (MIP). This process is called ‘aging’ and results in conversion of the DFP-inhibited serine hydrolase labeled at its active site from a reactivatable form (i.e., by treatment with nucleophilic agents) to a non-reactivatable form (Berends et al. 1959; Jansz et al. 1959). Loss of an isopropyl group would also cause proteolysis-independent loss of label from the protein-bound DIP, and may complicate interpretation of label clearance rates, depending on the relative rate in shunted and control rats. To evaluate the possibility of ‘aging’, samples of the brain subcellular fractions P_1 + P_2 and S_2 from three control and three 8–9-week shunted rats (rats from experiment 2 in Table 5 that were killed at 73h after [^{3}H]DFP and 1h after [^{14}C]DFP) were hydrolyzed in NaOH to release DIP and MIP from protein, and subjected to ascending paper chromatography in butanol:acetic acid:water (4:1:5) (Berends et al. 1959; Jansz et al. 1959). Preliminary experiments established the positions of labeled DIP and MIP by autoradiography. For quantification of tissue samples, the chromatograms were dried, each lane was cut into 1 cm strips that were placed in liquid scintillation vials, labeled compounds were eluted from the paper with water, and ^{3}H and ^{14}C levels were determined by liquid scintillation counting. There was some ‘aging’ of the [^{3}H]DFP at 73h after the injection, but less, if any, ‘aging’ of [^{14}C]DFP at 1h after injection (Table 6). Most important is the finding that the extent of ‘aging’ was similar in control and shunted rats. Thus, ‘aging’ contributes to elimination of ^{3}H from tissue, but it should not interfere with the use of ^{14}C as a relative internal standard at1h after the injection and it would not contribute to differences in label clearance between control and hyperammonemic shunted rats.

DISCUSSION

Increased levels of ammonia in body tissues may reduce lysosomal function by raising intralysosomal pH due to diffusion of NH_3 into these organelles followed by protonation and trapping of NH_4^+ (Seglen 1983; Lüllmann-Rauch 1979). The major goal of the present study was to determine whether proteolytic capacity is reduced after portacaval shunting. Protein degradation was estimated by determination of clearance of label from (i) serine esterases and proteases that were covalently labeled at the active site with a ‘suicide inhibitor’, DFP, and (ii) other proteins that are labeled by DFP on tyrosine or other moieties (Casida and Quistad 2005; Schopfer et al. 2010). This approach labels specific classes of proteins using a procedure that is independent of protein synthesis and not influenced by recycling of labeled amino acids. The major finding is that DFP label clearance was reduced in many, but not all, samples from portacaval-shunted compared with control rats, consistent with diminished capacity for protein clearance. These findings extend the approach used by others using DFP to evaluate turnover of serine esterases and proteases and to map their

localization (see Introduction). Reduced label clearance and increased labeling of protein by DFP in shunted rats support future investigations to evaluate the extent to which lysosomal functional deficits contribute compensatory responses of target protein levels in shunted rats and to morphological changes in brain cells after portacaval shunting or ammonia treatment, such as membrane and lipid accumulation (see Introduction). Disruption of vesicular and protein trafficking among the Golgi cisternae by the weak base chloroquine alters the distribution of mannose-6-phosphate receptor (Brown et al. 1984), suggesting that intracellular trafficking of various proteins and cell-surface receptors may be also disrupted in organs of hyperammonemic rats.

Characterization of the use of labeled DFP to evaluate proteolytic capacity showed that protein labeling is rapid, very small amounts of the injected label are covalently bound to brain protein, tracer amounts of DFP do not measurably inhibit acetylcholinesterase, and subcellular distribution does not parallel that of acetylcholinesterase, indicating that other proteins are the major targets of DFP in brain. Label clearance is relatively fast, with about 50% being lost within a 24h interval, and reutilization of labeled metabolites of diisopropylphosphate is negligible. There are, however, some limitations of the use of DFP and other organophosphates to evaluate proteolytic capacity. First, ‘aging’ will cause loss of label independent of proteolysis when the DFP is labeled in the isopropyl moieties. Use of ^{32}P- or ^{33}P-DFP for the first label in double-label assays would be preferable since the P-label would not be lost by ‘aging’, but unfortunately phosphate-labeled DFP was not commercially available. Evaluation of the proteins labeled by DFP was beyond the scope of the present study, but previous studies have shown that ‘aging’ varies among serine hydrolases (‘aging’ is low for chymotrypsin, trypsin, and liver ali-esterase compared with pseudocholinesterase), and with pH, temperature, and labeling agent (e.g., (Jansz et al. 1959; Clothier and Johnson 1979; Coult et al. 1966; Green and Nicholls 1959) and references cited therein). Second, clearance of label from DFP-target proteins only assays a specific class of proteins, and overall protein degradation and non-lysosomal proteolysis need to be examined. The results of the present study are consistent with impaired lysosomal function, but that needs to be proven directly. Future studies need to demonstrate accumulation of labeled material in lysosomes when function is impaired by weak bases, as previously shown by Gerard and colleagues (1988; 1979; 1977) for [^{14}C]NEM-labeled muscle protein. Third, much higher labeling by DFP of the meninges and choroid plexus compared with brain parenchyma may give these structures a greater influence on the overall apparent label clearance. Evaluation of label clearance from specific proteins after separation by gel electrophoresis would enable identification of proteins affected by portacaval shunting, but this approach may be influenced to a greater extent by ‘aging’, which would have to be evaluated for each protein of interest. Alternative protein labeling reagents may be useful to minimize the disadvantages of DFP or to extend the classes of protein being assayed. For example, labeling of the surface of the protein rather than the active site may cause the protein to be recognized as abnormal and targeted for more rapid degradation (Opresko et al. 1980), but this would not be an issue if lysosomal degradative capacity is being examined. To summarize, use of DFP to label specific proteins has both advantages and disadvantages, with ‘aging’ being a potentially serious issue that must be taken into account.

Important factors that can influence brain protein turnover, blood-brain barrier transport, and energy metabolism in portacaval-shunted rats include blood and brain ammonia levels, brain glutamine content, and plasma and brain glucose levels. Levels of all of these compounds are influenced by the procedure used to construct the portacaval shunt, and comparison of literature studies revealed that the glue method produces rats with much higher brain ammonia and glutamine levels and lower plasma and brain glucose levels (see Table 6 in Cruz and Dienel (1994)). The animal model used in the present study consistently produces brain ammonia levels in the range of 0.37 to 0.67 μmol/g and relatively normal and stable brain glucose levels within the range of 1.9-2.2 μmol/g between 1–12 weeks after shunting ((Cruz and Dienel 1994) and references cited therein). These rats have reduced rates of glucose utilization (CMR_glc) at one week after shunting, followed by progressive increases with time, with many brain regions attaining near-normal CMR_glc at 8–12 weeks (Cruz and Duffy 1983). Although metabolic fluxes in many pathways are altered by portacaval shunting, the rats used in the present study are not expected to incur energy failure or other serious consequences of much higher ammonia levels.

Effects of portacaval shunting on incorporation of three labeled amino acids into brain protein in the present study replicated some, but not all findings, in previous reports in the literature. Reduced label incorporation into brain protein was observed with lysine and phenylalanine but not with valine, whereas label incorporation into liver protein was reduced with lysine but increased with phenylalanine and valine (Tables 1 and 2). Wasterlain et al. (1978) reported a larger reduction (50%) in lysine incorporation into brain protein, but no effect in liver in 8-week shunted rats. Dunlop et al. (1984) observed no change in labeling of protein in brain, liver, muscle, or testicle with valine flooding of 4-week shunted rats. Other studies using loading or tracer doses different labeled amino acids reported either reduced (Lundborg and Hamberger 1977; Helewski and Konecki 1994) or unchanged (Cremer et al. 1977) label incorporation into protein in brain and other organs at various times after portacaval shunting. Because the apparently-discrepant findings with different amino acids in brain and liver in the present study were obtained in the same animal model and same assay conditions, contributions of varying ammonia level and other pathophysiological changes in the shunted rats can be ruled out, whereas these factors may contribute to discordant findings in the literature. Flooding of the brain precursor pool with valine was more effective than with lysine or phenylalanine (Tables 1 and 2), and a likely explanation for differences found with the three amino acids in brain and liver in our study is incomplete equilibration of the aminoacyl-tRNA specific activity with the precursor amino acid pools in plasma and tissue. Valine is, as concluded by Dunlop et al. (1984), considered to be least likely to be affected by differences in amino acid reutilization, blood and tissue pool sizes, blood-brain barrier transport, or metabolism that occur after shunting (Mans et al. 1984; Mans et al. 1982; Jeppsson et al. 1983; Jeppsson et al. 1979; James et al. 1978). In normal rats, large doses of valine do reduce the contribution of recycling of amino acids from protein degradation back into the protein synthesis pathway, but they only raise the ratio of brain valyl-tRNA specific activity to that of plasma valine to 0.73 compared with 0.92 for liver (Smith and Sun 1995; Smith et al. 1991). Thus, recycling, transport, and metabolism may still influence the outcome of in vivo precursor flooding assays of protein synthesis in various tissues of the shunted rats. Calculation of rates of protein synthesis during flooding

experiments based on the specific activity of the acid-soluble pool in tissue is, therefore, unlikely to be as accurate as desired, but results obtained with flooding doses of valine are probably most reliable. To resolve the apparent discrepancies in amino acid incorporation with different precursors, it will be necessary to determine the specific activities of aminoacyl-tRNAs compared with the specific activities of the amino acids in plasma and tissue for each precursor. Nevertheless, the fact that there are differences among shunted and control rats with respect to labeling of the acid-soluble pools and amino acid incorporation into protein (Tables 1 and 2) is of interest because the findings indicate that disruption of transport, metabolism, and pool mixing in shunted rats are of sufficient magnitude to affect calculated rates of protein synthesis under flooding conditions.

In conclusion, covalent protein-labeling reagents, such as DFP, are useful for evaluation of the capacity for proteolysis under pathophysiological conditions, and future studies can use these reagents to identify the labeled proteins and their cellular and subcellular localization. In portacaval shunted rats, the slower clearance of DFP-labeled target proteins and the increase in the amount of these proteins in brain of portacaval-shunted rats are associated with small, if any, changes in brain protein synthesis, based on valine label incorporation. Dysfunction of lysosomes and other acidic organelles that are affected by chronic hyperammonemia would reduce removal of proteins and lipids throughout the body and may contribute more to the progressive structural abnormalities in brain than disruption of protein synthesis. Therapeutic strategies that reduce ammonia levels would be expected to help normalize protein and lipid turnover and trafficking.

Acknowledgments

This work was supported, in part, by National Institutes of Health grants DK16739 and DK081936. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the National Institutes of Health. The funding sources had no role in study design; collection, analysis, and interpretation of data; writing of the report; and the decision to submit the article for publication.

Abbreviations

CMR_{glc}	local rate of glucose utilization
DFP	diisopropylfluorophosphate
DIP	diisopropylphosphate
MIP	monoisopropylphosphate
NEM	N-ethylmaleimide
PCS	portacaval shunt
TCA	trichloroacetic acid

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Table 1

Incorporation of [^{3}H]lysine and [^{14}C]phenylalanine into brain and liver protein at 8 weeks after portacaval shunting

Amino acid and treatment	Body wt. (g)	TCA-soluble fraction (nCi/g wet wt./h)				Amino acid incorporation (pCi/mg dry wt. protein/h)			
		Cerebral cortex		Liver		Cerebral cortex		Liver	
[^{3}H]Lysine			%		%		%		%
Controls	402 ± 15	162 ± 13 (47.3 ± 5.7)	100 (100)	960 ± 64 (748 ± 14)	100 (100)	83.3 ± 1.6	100	255.6 ± 50	100
PC-shunt	392 ± 50	139 ± 10 (38.6 ± 1.2)	86 (82)	2080 ± 250^c (1801 ± 19^c)	217 (241)	71.2 ± 6.7^a	86	109.4 ± 12.8^b	43
[^{14}C]Phenylalanine									
Controls	394 ± 10	2.81 ± 0.32	100	4.57 ± 1.01	100	14.4 ± 0.6	100	32.1 ± 6.8	100
PC-shunt	340 ± 60	5.18 ± 0.45^c	184	8.87 ± 1.05^b	194	11.2 ± 0.5^b	78	46.8 ± 9.5	146

Control and 8-week portacaval (PC)-shunted rats were injected intraperitoneally with L-[3,4-^{3}H(N)]lysine (10 mmol/kg, 50 μCi/mmol) or with L-[alanine-1-^{14}C]phenylalanine (5 mmol/kg, 8.27 μCi/mmol) and killed 1h later. The trichloroacetic acid (TCA)-soluble fraction represents the label in the precursor pool; two values are listed for [^{3}H]lysine, the total ^{3}H in the acid-soluble fraction and the non-volatile fraction (in parentheses) that is assumed to represent mainly [^{3}H]lysine (see Methods). Values are means ± SD (n = 6/group); percentages of respective control values were calculated with mean values.

^{a}P<0.05,

^{b}P<0.01,

^{c}P<0.001 vs. control (t-test).

Table 2

[^{14}C]Valine accumulation and incorporation into protein in control and portacaval-shunted rats

Treatment	n	Body wt. (g)	TCA-soluble fraction (nCi/g wet wt./h)						Valine incorporation into protein [a] (pCi/mg dry wt. protein/h)					
			Brain		Liver		Kidney		Brain		Liver		Kidney	
				%		%		%		%		%		%
Control	7	381 ± 14	3.8 ± 0.2	100	14.1 ± 0.6	100	13.5 ± 0.8	100	4.1 ± 0.3	100	15.8 ± 1.3	100	12.4 ± 0.9	100
PC-shunt														
3 days	5	237 ± 7	7.8 ± 0.4[b]	205	19.8 ± 1.1	140	13.4 ± 0.8	99	4.5 ± 0.1	110	25.7 ± 0.8[b]	162	16.5 ± 0.9[a]	133
2 weeks	4	272 ± 72	(5.4 ± 0.6[a])[c]	(142)	23.9 ± 6.2	170	11.8 ± 0.9	87	(5.8 ± 0.3[b])[c]	(141)	34.4 ± 1.4[b]	218	17.0 ± 1.6[a]	137
8 weeks	5	422 ± 28	5.6 ± 0.4[a]	147	16.5 ± 1.4	117	13.2 ± 0.5	98	4.0 ± 0.1	98	24.3 ± 1.8[a]	154	13.8 ± 0.5	111
12 weeks	5	407 ± 30	6.0 ± 0.2[a]	158	36.9 ± 4.8[a]	262	14.7 ± 1.1	109	4.7 ± 0.2	115	21.8 ± 0.6[a]	138	13.8 ± 0.2	111

Rats were injected intraperitoneally with L-[1-^{14}C]valine (7.5 mmol/kg, 2.66 μCi/mmol) at intervals after portacaval (PC) shunting, and the label in the precursor pool (TCA-soluble fraction) and protein determined 1h later (see Methods). Values are means ± SD; percentages of respective control values were calculated with mean values.

[a] $P<0.05$,

[b] $P<0.01$ vs. control, ANOVA and Dunnett's test.

[c] Values for brain in 2-week shunts were for cerebral cortex; all others represent whole brain.

Table 3

Loss of ^{3}H from [^{3}H]DFP-labeled brain protein

Time after labeling	Acid-insoluble ^{3}H in whole homogenate	
	(DPM/g wet wt.)	(Percent)
4h	226,075 ± 51,718	100
24h	94,027 ± 13,732	42
48h	63,037 ± 23,226	28

Control rats were killed at intervals after intravenous injections of 300 μCi [^{3}H]DFP/kg, brains were removed and homogenized in 0.32 M sucrose, and the acid-insoluble ^{3}H contents of the whole homogenate and subcellular fractions were assayed. Values are means ± SD (n = 3/group). The slope of the regression of the plot of log(dpm in whole homogenate/g tissue) vs. time is $y = -0.014x + 5.487$ ($r^2 = 0.998$). Release of label from the $P_1 + P_2$, P_3, and S_3 subcellular fractions at 24h compared with 4h was similar to that of whole homogenate, with the percentages of label remaining at 24h ranging from 44–47%.

Table 4

Tracer doses of DFP do not cause measurable inhibition of acetylcholinesterase

Treatment	Brain acetylcholinesterase activity (μmol/min/g wet wt.)
Controls	10.8 ± 0.1 (7)
Portacaval shunt (8–9 weeks)	10.6 ± 0.2 (3)
DFP-treated controls	
0.008 mg/kg	11.2 ± 0.6 (6)
0.010 mg/kg	10.5 ± 0.3 (3)
0.1 mg/kg	9.8 ± 0.3 (5)
0.67 mg/kg	1.5 (1)

Rats were killed at 4h after intravenous injection of DFP, and acetylcholinesterase activity was measured in the whole homogenate. Values are means ± SD (n). 0.008 mg DFP/kg corresponds to 300 μCi [^{3}H]DFP/kg, and 0.025 and 0.05 mg DFP/kg correspond to 15 and 30 μCi [^{14}C]DFP/kg, respectively.

Table 5

$^{3}H/^{14}C$ ratios in DFP-labeled protein in control and portacaval-shunted rats

Treatment	$^{3}H/^{14}C$ ratio (mean ± SD)									
	Brain						Liver		Kidney	
	Homogenate		$P_1 + P_2$		S_2					
Experiment 1		%		%		%		%		%
Control (n=5)	6.6 ± 0.9	100	6.8 ± 0.9	100	6.3 ± 0.9	100	3.3 ± 0.6	100	4.1 ± 1.0	100
4–8 week PCS (n=3)	9.2 ± 1.1[a]	139	9.5 ± 0.9[b]	140	8.4 ± 1.0	133	7.5 ± 2.1[b]	227	6.7 ± 0.8	163
Experiment 2										
Control (n=5)	--		4.7 ± 0.5	100	3.2 ± 0.3	100	4.9 ± 0.7	100	4.8 ± 0.1	100
8–9 week PCS (n=5)	--		7.8 ± 1.0[a]	166	6.1 ± 0.9[a]	191	12.3 ± 3.7[a]	251	11.3 ± 1.4[a]	235
Experiment 3										
Control (n=3)	1.8 ± 0.2	100	--		--		0.5 ± 0.0	100	1.1 ± 0.1	100
During first week after PCS (n=7)	2.7 ± 0.2[b]	150	--		--		1.5 ± 0.4[b]	300	1.5 ± 0.1	136
Experiment 4										
Control (n=10)	4.0 ± 0.4	100	--		--		2.0 ± 0.4	100	3.4 ± 0.4	100
2 week PCS (n=3)	6.8 ± 0.9[a]	170	--		--		1.9 ± 0.2	95	5.8 ± 0.7	171
4 week PCS (n=5)	4.7 ± 0.6	118	--		--		3.7 ± 0.8	185	4.6 ± 1.0	135
12 week PCS (n=5)	6.3 ± 0.3[a]	158	--		--		3.2 ± 0.4	160	6.7 ± 0.9[a]	197

$P_1 + P_2$ is the particulate fraction (cell debris, nuclei, mitochondria, synaptosomes, and myelin) obtained by centrifugation at 17,000g for 20 min. S_2 is the supernatant fraction containing the microsomal fraction (P_3) and cell-soluble fraction (S_3). In experiment 1, control and portacaval-shunted (PCS) rats were injected intravenously with [^{3}H]DFP (300 μCi/kg) followed 24h later by [^{14}C]DFP (30 μCi/kg). They were killed 24 h after the second injection and $^{3}H/^{14}C$ ratios determined in acid insoluble fractions. [a]P=0.058, [b]P<0.05 (t-test). In experiments 2 and 3 ([a]P<0.05, [b]P<0.01, t-test) and experiment 4 ([a]P<0.01, ANOVA and Dunnett's test) the control and PCS rats were injected with [^{3}H]DFP (300 μCi/kg) followed 72h later by [^{14}C]DFP (30 μCi/kg in experiment 3, and 15 μCi/kg in experiments 2 and 4), killed 1h after the second injection, and $^{3}H/^{14}C$ ratios determined. Each experiment used different batches of labeled DFP. Assays during the first week after shunting were initiated on day 3 by

injection of [^{3}H]DFP, followed by [^{14}C]DFP on day 6. To minimize stressing the newly-shunted rats, they were anesthetized for 10–15 min with ether during the injections; all other rats were injected while awake. In experiment 4, controls were assayed with each group of shunts, and data from all controls were pooled; multiple comparisons against a single control value in experiment 4 reduced sensitivity to detect significant differences. Percentages of respective control values were calculated with mean values.

Table 6

‘Aging’ of protein-bound DFP in brain of control and portacaval-shunted (PCS) rats

	^{3}H at 73 hours				^{14}C at 1 hour			
	$P_1 + P_2$		S_2		$P_1 + P_2$		S_2	
	% MIP	%DIP	% MIP	%DIP	% MIP	%DIP	% MIP	%DIP
Controls	12.4 ± 5.6	87.6 ± 5.6	9.9 ± 5.3	90.1 ± 5.3	3.2 ± 2.1	96.8 ± 2.1	1.1 ± 1.1	98.9 ± 1.1
8–9-week PCS	17.3 ± 8.4	82.7 ± 8.4	6.5 ± 0.9	93.5 ± 0.9	7.8 ± 5.4	92.2 ± 5.4	0.4 ± 0.7	99.6 ± 0.7

Values are means ± SD (n = 3/group). Rats were injected were injected intravenously with [^{3}H]DFP (300 μCi/kg) followed 72h later by [^{14}C]DFP (15 μCi/kg), killed 1 h after the second injection, subcellular fractions of brain were prepared from each rat, and protein precipitated with TCA. Acid-insoluble fractions were incubated with 1 N NaOH at 100°C for 1.5h, protein was precipitated, and soluble fractions were neutralized, concentrated, and subjected to ascending paper chromatography in butanol:acetic acid:water (4:1:5) to separate monoisopropylphosphate (MIP) and diisopropylphosphate (DIP) (see Methods and Results text).