Effect of glutamine on glutathione kinetics in vivo in dogs
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Abstract
To determine whether glutamine affects glutathione (GSH, γ-glutamyl-cysteinyl-glycine) metabolism, seven healthy beagle dogs received 6-h infusions of [\textsuperscript{15}N]glutamate and [\textsuperscript{13}C]leucine after a 3-day fast. Isotope infusions were performed during oral feeding with an elemental regimen, supplemented with either L-glutamine or an isonitrogenous amino acid mixture, on two separate days and in randomized order. Timed blood samples were obtained, and a surgical duodenal biopsy was performed after 6 h of isotope infusion. GSH fractional synthesis rate (FSR) was assessed from [\textsuperscript{15}N]glutamate incorporation into blood and gut GSH, and duodenal protein synthesis from [\textsuperscript{13}C]leucine incorporation into gut protein. Glutamine supplementation failed to alter erythrocyte GSH concentration (2189 ± 86 vs. 1994 ± 102 A mol L\textsuperscript{−1} for glutamine vs. control; ns) or FSR (64 ± 17% vs. 74 ± 20% day\textsuperscript{−1}; ns). In the duodenum, glutamine supplementation was associated with a 92% rise in reduced/oxidized GSH ratio (P = .024) and with a 44% decline in GSH FSR (96 ± 15% day\textsuperscript{−1} vs. 170 ± 18% day\textsuperscript{−1}; P = .005), whereas total GSH concentration remained unchanged (808 ± 154 vs. 740 ± 127 A mol kg\textsuperscript{−1}; P = .779). We conclude that, in dogs receiving enteral nutrition after a 3-day fast: (1) glutamine availability does not affect blood GSH, and, (2) in contrast, in the duodenum, the preserved GSH pool, along with a decreased synthesis rate, suggests that glutamine may maintain GSH pool and intestinal redox status by acutely decreasing GSH utilization.
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1. Introduction
The tripeptide glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is present in millimolar concentrations in most tissues and plays a prominent role in the defense against oxidative stress and in the detoxification process of xenobiotics. Due to its –SH group, GSH indeed can reduce reactive oxygen species such as hydrogen peroxide [a process in which GSH is oxidized to the dimer glutathione disulfide (GSSG; oxidized GSH)] or can bind and inactivate noxious agents [1]. As the small intestine is a barrier tissue exposed to a variety of insults from the environment, maintenance of gut GSH is critical for intestinal function, and inhibition of its synthesis is associated with lethal intestinal damage in rodents [2]. Similarly, in red blood cells (RBCs), GSH helps maintain iron in its reduced form — a prerequisite for hemoglobin’s function — in spite of the high oxygen concentration prevailing in the blood. GSH depletion has been known to occur in various disease states, such as human immunodeficiency virus infection [3,4], inflammatory bowel disease [5,6], diabetes [7] or critical illness [8]. As GSH turnover is extremely rapid, ranging between 50% and 100% per day in human blood [4,7,9,10] to >100% per day in porcine gut [11,12], depletion of blood GSH could result from: (1) a decreased rate of synthesis; (2) an increased rate of utilization; or (3) a combination of the two. The rate of GSH synthesis can be assessed in vivo by monitoring the incorporation of a labeled precursor amino acid into newly formed blood GSH during the course of a stable isotope-labeled amino acid infusion [4,7,9,11,12]. In preliminary studies, we observed that fasting was associated with a decline in GSH concentration in RBCs [13] and the duodenum (unpublished data), a finding consistent with observations in a variety of species and tissues [10,11,14]. GSH synthesis and regeneration require

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reduced nicotinamide adenine dinucleotide phosphate (NADPH) and precursor amino acids glutamate, cysteine and glycine. Amino acid supply could therefore play a major role in the regulation of GSH availability. Several lines of evidence point to a possible relationship between glutamine availability and GSH stores in the muscle, liver and gut. Muscle glutamine depletion indeed is a hallmark of severe disease states and, in critically ill patients, depletion of muscle GSH stores correlates with the loss of muscle function. Muscle glutamine supplementation attenuates the loss of skeletal muscle GSH [15]. Similarly, glutamine administration preserved liver GSH in rats receiving the cytotoxic drug 5-fluorouracil [16]; rises in blood GSH concentration and in GSH export from the gut were observed in rats receiving glutamine [17]. To our knowledge, the relationship between glutamine supply and blood or gut GSH has not been tested in either large animals or humans.

Intravenous glutamine has been shown to preserve nitrogen balance after major surgery [18,19] or bone marrow transplantation [20], whereas enteral glutamine has been shown to decrease leucine oxidation, an index of protein wasting, in healthy adults [21], children with muscular dystrophy [22], preterm infants [23] or dexamethasone-treated dogs [24]. Although the small intestine is a prominent site of glutamine uptake, the effect of glutamine on gut protein synthesis, however, remains debatable since some studies have suggested increased gut protein synthesis during glutamine supplementation [24,25], while other studies have not [26]. The aim of this study was to determine whether a short-term oral glutamine administration would acutely modulate duodenal protein synthesis and GSH metabolism in erythrocytes and the duodenum. This question was addressed in dogs that were refed via enteral nutrition after a 3-day fast to mimic underfeeding commonly associated with acute illness in humans.

2. Materials and methods

2.1. Experimental design

All studies were conducted in accordance with current guidelines from the French Department of Agriculture for the use of animals in biological research. Seven adult beagle (four female, three male) dogs were studied. Each animal underwent two isotope infusion studies (as described below) at least 3 months apart. Only healthy animals that had a good appetite, had normal stools, had normal body temperature (38.5–39.5°C) and were not on medications were enrolled.

Each dog was studied after a 3-day starvation period, which was designed to mimic the decreased dietary intake that occurs in severe illness. On isotope infusion day, each dog was bottle-fed small (≈40 ml) aliquots of a complete liquid nutrient mixture at 20-min intervals throughout the infusion period, at a rate supplying 1/24 of their regular daily intake per hour. The enteral regimen was supplemented with: (1) L-glutamine (1561±22 μmol kg⁻¹ h⁻¹; Sigma-Aldrich, Steinheim, Germany) on one study day, and (2) an isonitrogenous glutamine-free amino acid mixture on the other study day. The order of regimens was randomized. On the day of each isotope infusion at 0830 h, each dog was weighed and two short intravenous catheters (Vasocan 20 gauge; B Braun Medical, Emmenbrücke, Germany) were placed using aseptic technique: one in the cephalic vein of the forelimb for isotope infusion and another one in the jugular vein for blood sampling. At 0845, 0850 and 0855 h, three baseline 5-ml blood samples were obtained to determine background isotope enrichment in RBC glutamate, glutamine and GSH. Starting at 0900 h, each dog received a primed 6.5-h continuous infusion of: (1) L-[1-13C]leucine (99% 13C, 7.5 μmol kg⁻¹ prime, 10.5±0.3 μmol kg⁻¹ h⁻¹ infusion rate; Mass Trace, Woburn, MA, USA); and (2) L-[15N]glutamic acid (99% 15N, 45 μmol kg⁻¹ prime, 45.6±1.0 μmol kg⁻¹ h⁻¹ infusion rate; Cambridge Isotope Laboratories, Andover, MA, USA).

Simultaneous with tracer infusion, dogs were bottle-fed a liquid diet at 20-min intervals throughout the isotope infusion study. Enteral diets were designed to mimic the dogs’ regular diets, and were isonitrogenous and isonitrogenous (Table 1). The elemental nutrient mixture was prepared using the contents of three-compartment bags (Clinomel-N5; generously provided by Baxter, Maurepas, France) comprising 25% D-glucose, 20% long-chain triglyceride/glycerol emulsion and a glutamine–glutamate-free crystalline amino acid mixture designed for parenteral nutrition. Dogs received either the Clinomel-N5 amino acid mixture as their sole source of amino acids on one study day (control day), or the Clinomel-N5 amino acid mixture plus natural L-glutamine (1560 μmol kg⁻¹ h⁻¹) on the other study day (glutamine day).

Five-milliliter venous blood samples were collected 90, 150, 210, 240, 270, 300 and 330 min after the start of isotope infusion. Tubes were kept on ice until centrifugation at 4°C at 5000×g for 10 min. Plasma was removed, and the pellet

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<td>Dietary intake during isotope infusion</td>
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Data are expressed as mean±S.E. for seven dogs.
was washed twice with saline. The volume of removed plasma was then replaced by the same volume of cold distilled water in order to achieve hemolysis of RBCs. After this step, samples were stored at −80 °C until analysis. Hematocrit was determined in duplicate for each blood sample.

During the last 30 min of tracer infusion (i.e., around 1500 h), a full-thickness duodenal tissue sample of 900 mg was surgically excised using a short midline laparotomy after a short general anesthesia with Imalgene1000 (Merial, Lyon, France). The biopsy sample was immediately rinsed with ice-cold 0.9% NaCl, blotted and split into four aliquots weighing 400, 250, 200 and 50 mg, respectively. Each piece was weighed and frozen at −80 °C until analysis.

2.2. GSH analysis

Both the concentration and the isotope enrichment of RBC GSH were assessed using gas chromatography/mass spectrometry (GC/MS), as described [13]. For duodenal samples, 400 mg of tissue was homogenized in 1 ml of 0.2 mol L−1 phosphate buffer with 0.6 μmol of homo-GSH (as an internal standard) and 160 μmol of dithiothreitol. The mixture was homogenized on ice, and 2 ml of 0.2 mol L−1 phosphate buffer was added before derivatization. RBCs and duodenal samples were derivatized for GC/MS analysis, as described [7, 13]. For GSH enrichment determination, a [15N]labeled GSH standard was produced in vitro as described [13]; on the day of analysis, an aliquot of the standard was thawed, derivatized at the same time as each series of tissue and blood samples, and used to obtain a standard curve for the assessment of the M+1/M ion ratio in GSH by GC/MS analysis.

2.3. Glutamate and glutamine analysis

RBC glutamine and glutamate concentrations, and [15N]glutamate enrichment were assessed as previously described [13]. For duodenal samples, 200 mg of tissue was transferred into a 10-ml plastic tube containing 1 ml of cold distilled water, 20 nmol of L-[U-13C]glutamine and 75 nmol of homoglutamate. The mixture was homogenized on ice and deproteinized by sulfoalicylic acid. The supernatant was adjusted to a pH of 3 and poured on top of a plastic microcolumn containing AG50 cation exchange resin (Sigma-Aldrich). After applying the sample, the column was rinsed with cold distilled water, and 2 ml of 6 mol L−1 ammonium hydroxide was added to each column to elute both glutamate and glutamine. The latter was derivatized to its heptfluorobutyramide propyl ester derivatives and analyzed by GC/MS as described [13].

2.4. Calculation of GSH kinetics

The fractional synthesis rate (FSRgsh rbc; % day−1) of erythrocyte GSH was calculated from the incorporation of labeled glutamate into RBC GSH:

\[
FSR_{gsh \ rbc} = 100 \times 24 \Delta E_{gsh \ rbc}/(\Delta t E_{gsh \ rbc}),
\]  

where \(\Delta E_{gsh \ rbc}\) is the rise in RBC [15N]GSH enrichment during time interval \(\Delta t\) (h) when RBC-free [15N]glutamate enrichment (\(E_{gsh \ rbc}\)) was at steady state (i.e., between the end of the second and the sixth hour of isotope infusion); 24 converts FSRgsh rbc from h−1 to day−1; and 100 converts a fraction of unity to percent. The FSR of duodenal GSH (FSRgsh gut) was calculated using the analogous equation:

\[
FSR_{gsh \ gut} = 100 \times 24 \Delta E_{gsh \ gut}/(\Delta t E_{gsh \ gut}),
\]  

where \(\Delta E_{gsh \ gut}\) is 15N enrichment in the GSH in the duodenum at time \(t\); \(E_{gsh \ gut}\) is the 15N enrichment of the precursor pool (intracellular free glutamate); and \(\Delta t\) is the time lap (h) between the start of tracer infusion and the time at which biopsy was obtained. The calculation assumed 15N enrichment in the intestinal GSH at time 0 to be zero. On the second [15N]glutamate infusion day, “contamination” by residual [15N]GSH enrichment from the previous isotope infusion was unlikely since (a) a rapid intestinal GSH turnover implied that duodenal isotopic GSH must return to its baseline levels within days after an isotope infusion, as was indeed observed in the case of erythrocyte GSH, and (b) the interval between isotope infusions exceeded 90 days.

As measurements were performed under conditions of steady state, GSH synthesis was balanced by the rate of GSH utilization during the 4 h of isotopic steady state or by GSH fractional turnover rate, and a single equation was used for the calculation of these parameters.

2.5. Determination of reduced/oxidized GSH ratio

For the determination of intestinal total (GSx), reduced (GSH) and oxidized (GSSG) GSH by enzymatic assay, an ≈ 50-mg duodenal sample was homogenized in phosphate buffer and centrifuged for 15 min at 12,000×g at 4°C. Supernatants were deproteinized with SSA and analyzed for GSH and GSSG content using the GSH disulfide reductase 5,5′-dithiobis-2-nitrobenzoic acid recycling method, as described by Tietze [27] and modified by Baker et al. [28].

2.6. Determination of duodenal protein FSR

Duodenal protein synthesis (FSRprot gut) was determined by measuring the incorporation of [13C]leucine into gut protein. Briefly, ≈ 280 mg of tissue was homogenized in 0.9% NaCl, and the protein was then precipitated with trichloroacetic acid. The supernatant containing intracellular free amino acids was removed and dried under nitrogen flux for the determination of [13C] enrichment in intracellular free leucine (precursor pool) by GC/MS, after derivatization of free leucine to its tert-butyl dimethylsilyl derivative [29]. Pellet protein was hydrolyzed, and its [13C]leucine enrichment was determined by GC–combustion–isotope ratio MS, as described [26, 30]. Duodenal protein FSR was calculated as:

\[
FSR_{prot \ gut} = 100 \times 24 \Delta E_{prot \ gut}/(\Delta t E_{leu \ gut}),
\]
3. Results

The dogs’ body weight did not change between experimental days (13.9±0.8 and 13.9±0.6 kg for control and glutamine days, respectively; \(P=.86\)).

3.1. Blood glutamine, glutamate and GSH

As shown in Fig. 1, intracellular erythrocyte \([^{15}\text{N}]\text{glutamic acid enrichment reached a plateau by the end of the second hour of tracer infusion, and} \([^{15}\text{N}]\text{GSH enrichment increased linearly between the third and the sixth hour of tracer infusion.}\)

Although oral glutamine supplementation resulted in a significant 87% rise in RBC glutamine concentration (\(P=.016\), this was not associated with any change in glutamate (Table 2) or total GSH concentrations (2189±86 vs. 1994±102 \(\mu\text{mol L}^{-1}\) for glutamine vs. control, respectively; \(P=.098\)) (Fig. 1). RBC GSH synthesis was not affected by glutamine supplementation (64±17% day\(^{-1}\) vs. 74±20% day\(^{-1}\) for glutamine vs. control, respectively; \(P=.772\) (Fig. 1).)

3.2. Duodenal glutamine, glutamate and GSH

In the duodenum, glutamine supplementation induced a 213% rise in free glutamate concentration (\(P=.003\); the slight increase in duodenal glutamate level failed to reach statistical significance (Table 2). Although enteral glutamine failed to alter total GSH level ([GSx], 0.808±0.154 vs. 0.740±0.127 \(\mu\text{mol g}^{-1}\) wet tissue for glutamine vs. control, respectively; \(P=.779\), enteral glutamine supply was associated with a 44% decline in GSH synthesis (96±15% of control)."

![Fig. 1. Erythrocyte GSH metabolism. Erythrocyte GSH concentrations (mmol L\(^{-1}\); bottom panel), time course of RBC \([^{15}\text{N}]\text{glutamate and} \([^{15}\text{N}]\text{GSH enrichment during an infusion of L-}^{[15]\text{N}}\text{glutamic acid (middle panel), and GSH FSR during continuous enteral nutrition: effects of glutamine-supplemented diet (solid symbols) vs. control diet (open symbols) (top panel). Data represent the mean±S.E. of seven dogs.}]

\[\Delta E_{\text{prot gut}}\] is the rise in \(^{13}\text{C}\) enrichment in bound leucine in duodenal protein between time 0 and the time of duodenal biopsy; \(E_{\text{leu gut}}\) is \(^{13}\text{C}\) enrichment of the precursor pool (intracellular free leucine); and \(\Delta t\) is the time elapsed (h) between the start of labeled leucine infusion and the performance of biopsy. \(\Delta E_{\text{prot gut}}\) was calculated as the difference in \(^{13}\text{C}\)leucine enrichment between duodenal protein-bound leucine at the time of biopsy and plasma protein-bound leucine at time 0.

2.7. Statistical analysis

Data are presented as mean±S.E. of seven dogs, except for intestinal GSH FSR and GSH/GSSG ratio, which were measured in six and five dogs, respectively. Data were compared between treatments using paired Wilcoxon or unpaired Mann–Whitney nonparametric tests, when appropriate. Significance was established at \(P<.05\).
day/C0 

F 

18% day/C0 

1 for glutamine vs. control, respectively; P = .005) (Fig. 2) and a 92% increase in duodenal GSH/GSSG ratio (1.35 \pm 0.16 vs. 0.70 \pm 0.08; P = .02) (Fig. 3). Even though samples were aliquoted and frozen immediately after biopsies had been obtained to minimize GSH oxidation, oxidation of GSH during subsequent sample processing could be excluded. Yet, had oxidation occurred during sample processing, it would most likely have affected samples obtained from both arms of the study, so that this potential artifact would not have altered the overall direction of the change between study days.

4. Discussion

The findings of the current study suggest that, in dogs refed after a 3-day fast, enteral glutamine supplementation affects GSH metabolism in the gut. Glutamine indeed acutely decreased duodenal GSH synthesis rate and increased the reduced GSH/oxidized GSH ratio without altering total GSH content (reduced GSH plus oxidized GSSG). In contrast, glutamine affected neither duodenal protein synthesis nor blood GSH metabolism. These findings suggest that glutamine may spare intracellular GSH stores by decreasing its rate of utilization and/or degradation in the gut (a tissue with high rates of glutamine utilization), whereas it fails to do so in tissues that do not actively use glutamine as fuel, such as erythrocytes.

Whereas the GSH levels assessed in RBCs in the current study are consistent with values reported in humans [4,7] and dogs [31], the GSH concentrations measured in the duodenum (0.740 \pm 0.127 \mu mol g \(^{-1}\) wet tissue) are lower than values reported in humans [5,6] or rats [14,32] (6.69 \pm 4.94 and 2.26 \mu mol g \(^{-1}\) wet tissue, respectively). Interspecies differences, as well as starvation prior to the study, may account for the difference since restriction of dietary intake is known to deplete GSH in most tissues (including gut) in pigs [11], rats [32] and humans [5,10].

Both \[^{13}\text{C}]\text{leucine}\ and \[^{15}\text{N}]\text{glutamate}\ tracers were administered as primed continuous infusions, and equations appropriate for steady state were used for calculating protein and GSH FSR. This relies on the assumption that isotope enrichment in the precursor pool was nearly constant throughout isotope infusion. As steady state was documented in the case of plasma \[^{13}\text{C}]\text{leucine}\ (data not shown) and RBC \[^{15}\text{N}]\text{glutamate}, we assumed that this was the case for gut mucosal intracellular enrichment as well.

Glutamine supplementation failed to affect GSH turnover in RBCs, despite an 87% rise in RBC glutamine,
when compared to an isonitrogenous, isocaloric, glutamine-free supplement. In contrast, in the duodenum, a tripling in intracellular glutamine had a significant impact, as it was associated with a 50% decline in GSH synthesis rate. The maintenance of gut GSH pool size despite the decline in GSH synthesis rate implies that GSH utilization was acutely inhibited by glutamine. This is consistent with the twofold rise in GSH/GSSG ratio (Fig. 3), resulting from a decrease in GSSG. Oxidized GSH (GSSG) indeed is known to diffuse more easily out of the cell than reduced GSH [33]; as a consequence, any decrease in GSSG tends to limit the “leak” of total GSH from the cell. Taken together, the data therefore suggest that, in the duodenum, glutamine may preserve GSH stores not by enhancing GSH synthesis but by sparing reduced GSH.

Tissue specificity in the effect of glutamine may arise from differences in the ability of tissues to use glutamine as a fuel. The activity of glutaminase, a mitochondrial enzyme [34], is higher in the gut than in any other tissue [35] and is negligible in RBCs, which are devoid of mitochondria. The provision of glutamine accordingly increased intracellular glutamate concentration in the duodenum, whereas it failed to do so in RBCs. Glutamine may thus affect GSH kinetics only in tissues that are capable of converting glutamine to glutamate. We further speculate that the sparing effect of glutamine on duodenal GSH may result from the use of glutamine as a source of NADPH. After glutamine is hydrolyzed to glutamate, the latter can indeed be converted to α-ketoglutarate, and α-ketoglutarate can subsequently enter the Krebs cycle and produce malate. Whereas malate oxidation proceeds to oxaloacetate in most tissues, in the small intestine, malate can be transported out of the mitochondria into the cytosol, where malic enzyme can convert malate to pyruvate while reducing NADP⁺ to NADPH [36]. High activities of malic enzyme have been found in canine duodenum [36]. Increased availability of α-ketoglutarate derived from glutamate metabolism could thus enhance the production of NADPH via malic enzyme. As NADPH is required for the reduction of GSH, the rise in cellular NADPH availability could favor the reduction of GSSG to GSH and therefore spare gut GSH. Such a mechanism would imply that glutamine could indirectly act as an “antioxidant” in the small intestine.

Finally, the lack of any protein-anabolic effect of glutamine may arise from the relatively short duration of the fast in the current study. Earlier studies indeed showed that a 42-h fast failed to affect whole-body and glut protein turnover in dogs [37].

In conclusion, after a 3-day fast in dogs, supplementation of enteral feeding with glutamine affects neither gut protein synthesis nor RBC GSH, but is associated with a sharp decline in GSH utilization and an improvement in GSH redox status. The current findings therefore support a role for glutamine in the preservation of reduced GSH in the gut under conditions mimicking decreased dietary intake accompanying severe illness.

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