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ORIGINAL ARTICLE

Cysteine fluxes across the portal-drained viscera of enterally fed minipigs: effect of an acute intestinal inflammation

Didier Rémond · Caroline Buffière · Corine Pouyet · Isabelle Papet · Dominique Dardevet · Isabelle Savary-Auzeloux · Gary Williamson · Magali Faure · Denis Breuillé

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Abstract Cysteine is considered as a conditionally indispensable amino acid. Its dietary supply should thus be increased when endogenous synthesis cannot meet metabolic need, such as during inflammatory diseases. However, studies in animal models suggest a high first-pass extraction of dietary cysteine by the intestine, limiting the interest for an oral supplementation. We investigated here unidirectional fluxes of cysteine across the portal-drained viscera (PDV) of multi-catheterized minipigs, using simultaneous intragastric L-[^{15}N] cysteine and intravenous L-[3,3D2] cysteine continuous infusions. We showed that in minipigs fed with an elemental enteral solution, cysteine first-pass extraction by the intestine is about 60% of the dietary supply, and that the PDV does not capture arterial cysteine. Beside dietary cysteine, the PDV release non-dietary cysteine (20% of the total cysteine release), which originates either from tissue metabolism or from reabsorption of endogenous secretion, such as glutathione (GSH) biliary excretion. Experimental ileitis induced by local administration of trinitrobenzene sulfonic acid, increased liver and ileal GSH fractional synthesis rate during the acute phase of inflammation, and increased whole body flux of cysteine. However, cysteine uptake and release by the PDV were not affected by ileitis, suggesting an adaptation of the intestinal sulfur amino acid metabolism in order to cover the additional requirement of cysteine linked to the increased GSH synthesis. We conclude that the small intestine sequesters large amounts of dietary cysteine during absorption, limiting its release into the bloodstream, and that the other tissues of the PDV (colon, stomach, pancreas, spleen) preferentially use circulating methionine or cysteine-containing peptides to cover their cysteine requirement.

Keywords Cysteine · Glutathione · Portal-drained viscera · Ileitis

Abbreviations

Cys	Free cysteine
CySS	Cystine
CySSP	Cysteine bound through disulfide linkage to proteins or peptides
FSR	Fractional synthesis rate
GSH	Glutathione
PDV	Portal-drained viscera
TCys	Cys + CySS + CySSP
TNBS	Trinitrobenzene sulfonic acid
dSI	Distal small intestine

Introduction

Glutathione (L-γ-glutamyl-cysteinyl-glycine; GSH) is the major water soluble antioxidant in animal cells, protecting them from reactive oxygen species. GSH also plays a role in xenobiotic detoxification, metabolism of various

D. Rémond · C. Buffière · C. Pouyet · I. Papet · D. Dardevet · I. Savary-Auzeloux
INRA de Clermont-Fd/Theix, UMR 1019,
Unité de Nutrition Humaine, 63000 Clermont-Ferrand, France

G. Williamson · M. Faure · D. Breuillé
Nutrition and Health Department,
Nestlé Research Center, Lausanne, Switzerland

D. Rémond (✉)
Unité de Nutrition Humaine, INRA de Clermont-Fd/Theix,
63122 St Genès-Champanelle, France
e-mail: didier.remond@clermont.inra.fr

molecules (leucotriens, prostaglandins, formaldehyde, methylglyoxal, nitric oxide,…) and regulation of expression and/or activation of 'oxidation-sensitive' transcriptional factors necessary for the antioxidant response (Wu et al. 2004). Tissue GSH synthesis increases during the acute phase of inflammation and infection (Malmezat et al. 2000). However, a low GSH status is generally observed in chronic diseases or recovery phase after acute injury. This depletion in GSH is probably a consequence of an accelerated rate of utilization, related to the oxidative stress, which is not counteracted by an increased synthesis, this synthesis being partly limited by substrate availability (Breuillé and Obled 2000). Indeed, GSH synthesis depends mainly on the availability of cysteine, generally considered as the limiting amino acid for its synthesis (Breuillé and Obled 2000). Due to the important protective and metabolic functions of GSH, the maintenance of its status in pathological situations is thus considered as an important nutritional target. In this context, a good knowledge of the quantitative significance of cysteine fluxes across the gastrointestinal tract is of importance, because cysteine sequestration at this level will decrease the availability of cysteine for GSH synthesis in other organs.

Cysteine is used for protein synthesis but is also involved in the production of other essential molecules that include glutathione, taurine, coenzyme A, and inorganic sulfur. It is considered a dietary dispensable amino acid that can be synthesized in the body from methionine and serine by transmethylation and transsulfuration. Measurements of portal net flux of cystine (the major form of circulating cysteine) suggested an extensive intestinal utilization of dietary cysteine (Bos et al. 2003), and a low bioavailability of dietary cysteine. Nevertheless, studies reporting cystine net fluxes in the splanchnic area are scarce, and there are no data in the literature on the net flux of total exchangeable cysteine (TCys), meaning the sum of cystine (CySS), free cysteine (Cys) and cysteine bound through disulfide linkage to proteins and peptides (CySSP). Cys undergoes rapid autoxidation to CySS during sampling and analysis; nevertheless appropriate methods showed that in plasma it contributes to about 10–15% of Cys + 2 × CySS (Lash and Jones 1985; Jones et al. 2002; Blanco et al. 2007). Furthermore, CySSP accounts for about 40% of TCys (Lash and Jones 1985). Taking into account all these forms of circulating cysteine, portal recovery of dietary cysteine could be greater than previously estimated from cystine portal net flux only. In addition, portal net flux is the balance between release and uptake by the portal-drained viscera, and a better estimation of the recovery would be done with unidirectional flux measurements.

The aim of the present study was to explore unidirectional fluxes of TCys across the gastrointestinal tract, using minipigs as an animal model, combining measurement of net fluxes across the portal-drained viscera with dual intravenous and enteral infusion of isotopically labeled cysteine. The effect of an acute intestinal inflammation on these fluxes was investigated in order to determine the impact of an increased TCys demand on the net and unidirectional fluxes of TCys across the gastrointestinal tract. The study is part of a project aiming to determine the impact of intestinal inflammation on amino acid use by the gastrointestinal tract; some data of this project has already been published by Rémond et al. (2009).

Materials and methods

All procedures were in accordance with the guidelines formulated by the European Community for the use of experimental animals (L358-86/609/EEC) and the study was approved by the Local Committee for Ethics in Animal Experimentation (CREEA d'Auvergne, Aubière, France).

Animals

The study involved eight adult Pitmann-Moore minipigs (CEGAV, Passais-la-Conception, France), 8–10 months old (25–30 kg of body weight). They were housed in individual pens (1 × 1.5 m) separated by Plexiglass walls in a ventilated room with controlled temperature (20–23°C).

At least 2 weeks before initiating the experiment, minipigs were surgically fitted with a blood flow probe around the portal vein (14 mm probe, A-series, Transonic Systems, Inc., Ithaca, NY, USA), with permanent catheters (polyvinyl chloride; 1.1 mm i.d., 1.9 mm o.d.) in the aorta, the portal vein (for portal flux measurements) and the inferior caval vein (for tracer infusion), with a cannula (silicone rubber; 12 mm i.d., 17 mm o.d.) inserted into the distal ileum (for TNBS-ileitis induction), and a gastric catheter (for enteral nutrition). Surgical procedures for implantation of all these equipments, as well as post-surgical cares have been previously described in detail by Rémond et al. (2009). In addition, during the same surgery, a catheter was inserted in the cranial mesenteric vein (for mesenteric flux measurements). The tip of this catheter was extended with silicone rubber tubing (Silastic®, Dow Corning Corporation, Midland, MI, USA; 0.76 mm i.d., 1.65 mm o.d.). The length of the silicone rubber tip was adjusted during surgery so that the junction with the polyethylene chloride catheter was at the insertion site. It was directly inserted in the main arcade of the cranial mesenteric vein and slid downstream into the vessel so that the tip of the catheter was about 2–4 cm upstream the junction with the ileocecocolic vein. At this level, the mesenteric vein drains the distal half-part of the small intestine, including the ileum segment submitted to

inflammation. Finally, a blood flow probe was implanted around the mesenteric artery (satellite of the cranial mesenteric vein) at the level of the tip of the mesenteric vein catheter (2.5 mm probe, S-series).

Ileitis was induced by intraluminal injection of trinitrobenzene sulfonic acid (TNBS) as previously described by Rémond et al. (2009). Briefly, in fasted minipigs, a tube fitted at its tip with two inflatable latex balloons at 20 cm intervals was slid into the intestine of anesthetized minipigs via the ileal cannula, and a solution of TNBS/ethanol was applied for 30 min to the ileal segment isolated by the two balloons. Inflammatory consequences of this treatment were described by Rémond et al. (2009).

Experimental protocol

Throughout the experimental period, minipigs were exclusively fed with a liquid diet, enterally infused into the gastric catheter via a swivel system connected to a pump. The daily diet was prepared by mixing Nestlé® f.a.a™ (40 mL/kg of body weight), which is a low fat, nutritionally complete free amino acid diet, with Nutriose FB® (Roquette, Lestrem, France; 6 g/L of Nestlé® f.a.a™), which provided soluble dietary fiber. The mixture was made up to 1.2 L with water. It was infused at a rate of 200 mL/h for 6 h (between 0900 and 1500 hours). The enteral feeding provided 2 g of protein equivalent kg^{-1} d^{-1}, 167.4 kJ kg^{-1} d^{-1}, and 50 mg of cystine kg^{-1} d^{-1}.

TCys fluxes were monitored 4 days before (day −4), and 3 days after (day +3) treatment, in four minipigs being subject, on day 0, to TNBS treatment (TNBS group), and four control minipigs which did not undergo ileitis induction, but were submitted to a control treatment including only the 1day-starvation period (Ctl group) (Fig. 1). On the days of sampling, before starting the enteral nutrition, a set of arterial and venous blood samples was collected for the determination of the baseline enrichments. Once the infusion of the enteral solution was started (0 min), after an initial bolus equivalent to 1 h of infusion, L-[3,3D2] cysteine and L-[^{15}N] cysteine (Cambridge Isotope Laboratories, Andover, MA, USA) in sterile saline was infused (6 mL/h) at a rate of 3.5 µmol kg^{-1} h^{-1} in the caval vein and the stomach, respectively. Blood samples were then simultaneously taken from the artery, the portal and the mesenteric vein at 60, 120, 210, 270, 300, 330, and 360 min (Fig. 2). Portal and mesenteric blood flow was continuously recorded during the sampling session with an ultrasonic transit time flowmeter (Transonic Systems INC.) interfaced with a computer for data acquisition.

At the end of the last sampling day, immediately after the last blood samples were taken, the minipigs were euthanized by intravenous injection of 125 mg/kg body weight of sodium pentobarbital (Doléthal®; Vetoquinol, Lure, France). The abdomen was opened, and the ileum was then rapidly excised, flushed with saline and opened. Two 20 cm segments were isolated: one corresponding to the inflamed area (referred as distal ileum), the other one being collected about one meter upstream (referred as proximal ileum). For both segments, mucosa was removed and frozen in liquid nitrogen. Furthermore, a liver sample was cut off and immediately frozen in liquid nitrogen. Tissue samples were stored at −80°C.

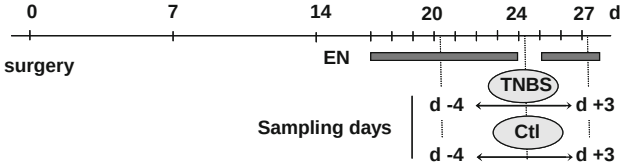

Fig. 1 Experimental design. Enteral nutrition (*EN*) started 3 days before the first sampling day (day −4). Two groups of minipigs were used: the TNBS group ($n = 4$) that undergoes ileitis induction on day 0, and the control group ($n = 4$) without ileitis induction

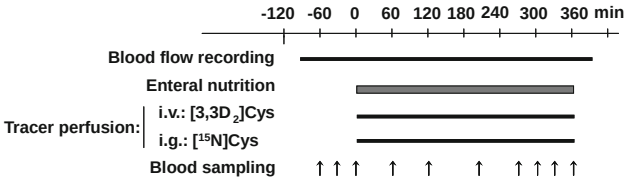

Fig. 2 Protocol design for a sampling day

Sample processing

Blood samples were collected in prechilled syringes (S-monovettes; Sarstedt, Nümbrecht, Germany) containing either EDTA-K (for whole blood analysis), or heparin lithium (for blood-gas analysis) as anticoagulant. Hemoglobin concentration was immediately determined using an automatic blood-gas analyzer (ABL510, Radiometer, Copenhagen, Denmark). Whole blood (1 mL) was mixed with 200 µL of phosphate buffer (pH 8.5) containing dithiothreitol (DTT 0.4 M) in order to reduce disulfide bonds, and the mixture was kept 15 min at room temperature for thiol reduction, before TCys determination (concentration and isotope enrichment). This preparation was deproteinized by the addition of 0.1 mL of sulfosalicylic acid (SSA, 50% w/v), the mixture was vortexed for 1 min, left 15 min at room temperature and then centrifuged at 10,000×*g* for 15 min at 4°C. The resulting supernatant was stored at −80°C.

Frozen powdered aliquots of tissue (0.1–0.2 g) were homogenized by sonication in 1.5 mL of phosphate buffer, pH 8.5, containing 5 mM EDTA and 0.08 mM DTT, and the mixture was kept for 15 min at room temperature. The reduction reaction was stopped by addition of 0.1 mL of SSA (50% (w/v). After centrifugation (10,000×*g*, 15 min, 4°C) the supernatant was stored at −80°C.

Analytical methods

Whole blood TCys concentration was measured in blood according to the method of Malloy et al. (1981). Blood and tissue total glutathione was determined by the enzymatic recycling procedure according to Robinson et al. (1992).

For isotopic enrichment of TCys and total glutathione, 80 µL of a solution containing 20 mM *N*-ethyl-maleimide (NEM), EDTA (2 mM pH 8.9) and SSA 2% (w/v), were added to 40 µL of supernatant and left 90 min at room temperature. The mixture was then diluted with 400 µL of water before analysis. The samples were analyzed by LC-MS/MS. Chromatographic separation was achieved on a Surveyor HPLC system (ThermoElectron, San Jose, CA, USA). Separations were carried out at 25°C using a 2.1 × 150 mm Acquity Atlantis dC18 column (Waters, Saint Quentin en Yvelines, France), with a particle size of 3 µm at a flow rate of 0.2 mL/min. Samples were eluted from the LC column using the following isocratic method: 0–22 min, 100% A [0.1% formic acid in water/methanol (95/5, v/v)]. Tandem mass spectrometry (MS/MS) analysis was carried out on a TSQ Quantum Ultra triple quadrupole mass spectrometer (ThermoElectron, San Jose, CA, USA), equipped with an electrospray source and operated in positive ion mode. The cysteine-NEM derivative was detected in single ion monitoring mode at $m/z = 247$ (M + 0), 248 (M + 1) and 249 (M + 2). The glutathione-NEM derivative was detected in multiple reaction scanning mode, using the transition of parent to daughter ions of 433–201 (M + 0), 434–202 (M + 1), and 435–203 (M + 2). Tracer/tracee ratio was converted to mole percent excess (MPE) using the enrichments observed in samples collected before tracer perfusion. For tissues, the tracer/tracee ratio of TCys and GSH at baseline was assumed to be the same as for blood TCys and GSH, respectively.

Calculations

TCys fluxes were calculated across the PDV and across the distal small intestine (dSI). Net and unidirectional fluxes were calculated using only the last four sampling times, from 300 to 390 min after the onset of enteral feeding and tracer perfusions, when amino acid concentrations, [^{15}N]cysteine and [D2]cysteine enrichment had reached a plateau (Fig. 3). Equations used to calculate TCys net and unidirectional fluxes across the PDV and the dSI, and to calculate the whole body flux of TCys are detailed in "Appendix".

For the reasons previously described by Jahoor et al. (1995), only estimates of the fractional synthesis rate (FSR, % day^{-1}) of GSH in liver and intestine mucosa can be obtained. FSR was calculated from cysteine isotopic enrichment in GSH of the tissue (Ei GSH, MPE), and the isotopic enrichment of TCys (Ei TCys, MPE) measured in three different pools, either in the tissue, or in the arterial blood, or in the venous drainage. Ei TCys was not constant during the entire infusion period and the plateau value was not reached immediately. The time course of Ei TCys can be described by a simple exponential: Ei TCys = Ei TCys max $(1-e^{-\lambda t})$, where Ei TCys max is the isotopic enrichment during the steady state and λ is an empirically fitted rate constant. The evolution of Ei TCys in whole blood can

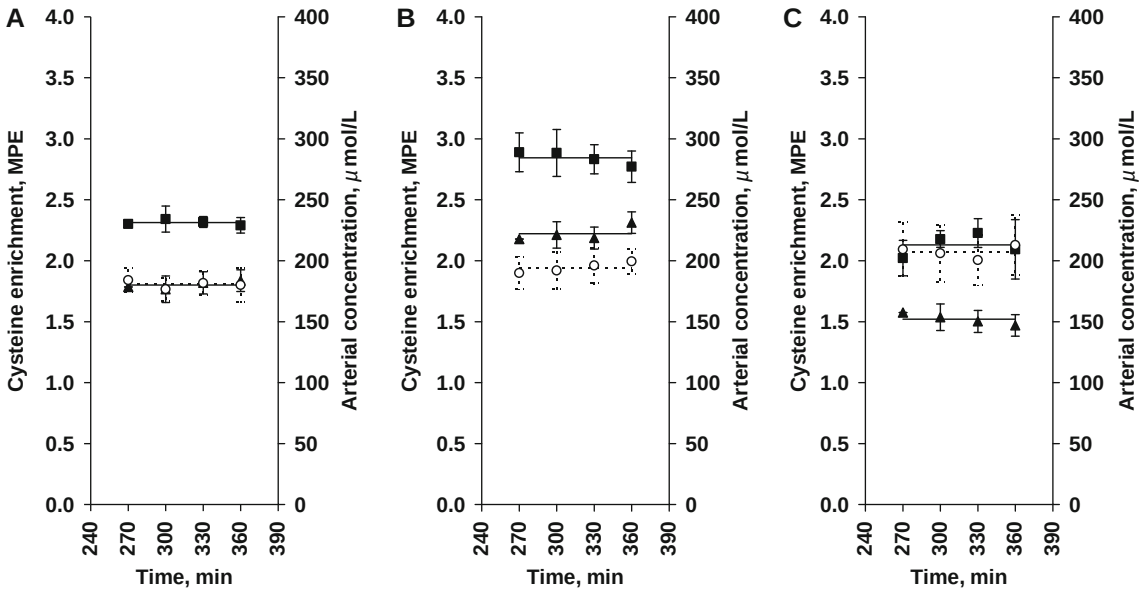

Fig. 3 Arterial TCys concentration (*open circles*), ^{2}H-enrichment (*filled squares*), and ^{15}N-enrichment (*filled triangles*) in minipigs receiving enteral feeding. Measurements were performed 4 days before (**a** $n = 8$), and 3 days after ileitis induction (**b** $n = 4$), at that time a control group was used (**c** $n = 4$)

also be described by a single exponential analogous to the equation presented above for tissues. We assumed that λ in tissue was equivalent to the value determined in blood, and FSR was calculated as:

$$\text{FSR} = \text{Ei GSH/Ei TCys} \times \lambda / \left[\text{t } \lambda - \left(1 - \text{e}^{-\lambda \text{t}}\right)\right]$$

FSR was calculated from both ^{15}N enrichment and ^{2}H cysteine isotopic enrichment.

Statistics

Data were analyzed using the repeated option of the PROC MIXED procedure of SAS (SAS/STAT Users Guide®, Release 8.1; SAS Institute Inc, Cary, NC, USA, 2000), with subjects as random effect and day, group, day × group as factors. When significant day × group interaction was found, the LSMEANS procedure was used to test differences at specific days, between and within groups. Unpaired *t* tests were used to compare, between the two groups, data obtained on concentrations, enrichments and FSR (day +3) in tissues. Values are means ± SEM. Values were considered different at $P < 0.05$ and considered as trends at $P < 0.1$.

Results

The arterial TCys and GSH concentrations (190 ± 9 and 424 ± 21 μM, respectively) were not affected by day of sampling or group, and no interaction between day and group was evidenced ($P > 0.10$). Whereas portal blood flow decreased between day −4 and day +3 in the control group, it increased in the TNBS group, and on day +3, portal blood flow was 70% greater ($P < 0.05$) in the TNBS group (39.3 ± 2.3 mL kg^{-1} min^{-1}) than in the control group (23.6 ± 2.0 mL kg^{-1} min^{-1}). Blood flow across the dSI accounted for about 10% of the portal blood flow and tended to be greater in the TNBS group after ileitis induction (2.47 ± 0.14 vs. 2.30 ± 0.14 mL kg^{-1} min^{-1}; $P = 0.088$). TCys net flux across the PDV and the dSI was not significantly affected by ileal inflammation (Table 1).

Table 1 Cysteine whole body flux, and cysteine fluxes across the portal-drained viscera and the distal small intestine in enterally fed minipigs 4 days before and 3 days after induction of ileitis

Item[a]	Day −4[b]	Day 3		ANOVA[c]		
		Control	TNBS	P_d	P_g	$P_{d \times g}$
Intragastric infusion						
Cysteine	68.9 ± 2.3	62.6 ± 4.1	67.3 ± 2.7	0.298	0.139	0.857
^{15}N-cysteine	3.5 ± 0.1	3.6 ± 0.0	3.5 ± 0.1	0.356	0.768	0.356
Whole body flux						
Ra_{iv}	145.5 ± 4.3	120.8 ± 6.4	158.3 ± 7.4	0.379	0.026	0.036
Ra_{ig}	188.1 ± 11.7	156.0 ± 2.1	221.5 ± 13.1	0.955	0.033	0.096
Portal-drained viscera fluxes						
F_{net} TCys	44.0 ± 5.8	37.6 ± 8.4	46.3 ± 7.2	0.707	0.831	0.083
Capt $TCys_{art}$	−0.41 ± 3.1	1.84 ± 6.5	6.6 ± 3.9	0.572	0.599	0.495
Capt $TCys_{lum}$	40.9 ± 4.8	33.6 ± 7.2	40.7 ± 5.5	0.611	0.323	0.975
Ap $TCys_{lum}$	31.5 ± 4.2	32.6 ± 9.3	30.0 ± 3.2	0.986	0.813	0.895
Ap $TCys_{endo}$	12.1 ± 4.4	6.9 ± 7.8	22.8 ± 8.5	0.753	0.783	0.129
Distal small intestine fluxes						
F_{net} TCys	6.0 ± 1.3	5.4 ± 1.2	9.0 ± 1.2	0.440	0.124	0.624
Capt $TCys_{art}$	−1.03 ± 1.3	−1.2 ± 1.3	4.4 ± 4.7	0.331	0.389	0.302
Ap $TCys_{lum}$	3.5 ± 0.8	5.0 ± 1.1	4.4 ± 0.9	0.201	0.450	0.695
Ap $TCys_{endo}$	1.5 ± 1.3	−0.8 ± 2.7	9.0 ± 4.3	0.352	0.063	0.269

Values are means (expressed as μmol kg^{-1} h^{-1}) ± SEM, $n = 4$ (day 3) or 8 (day −4)

F_{net} TCys cysteine net flux, *Capt $TCys_{art}$* arterial cysteine uptake, *Capt $TCys_{lum}$* luminal cysteine uptake, *Ap $TCys_{lum}$* luminal cysteine release, *Ap $TCys_{endo}$* non-luminal cysteine release = F_{net} TCys + Capt $TCys_{art}$−Ap $TCys_{lum}$

[a] Ra_{iv} and Ra_{ig}, cysteine whole body flux calculated from intravenous and intragastric tracer, respectively

[b] Because the data from the TNBS and Ctl groups were not different ($P > 0.10$) on day −4, only the averaged values of all animals are presented

[c] Data were analyzed by a mixed model ANOVA with subjects as random effect and day, group, day × group as factors. P_d, probability for a day affect; P_g, probability for a group effect; $P_{d \times g}$ probability for an interaction between days and groups

Regarding unidirectional fluxes of TCys, the dietary cysteine uptake by the PDV (Capt $TCys_{lum}$, which mainly involves the small intestine, where absorption takes place) was very high and corresponded to about 60% of the intragastric infusion (Table 1). The local inflammation induced by ileitis did not modify this high cysteine uptake. As a consequence, only about 40% of the dietary cysteine was released into the portal vein (Ap $TCys_{lum}$) and thus was available for hepatic metabolism. Moreover, dietary cysteine release by the dSI accounted for about 10% of the portal delivery of dietary cysteine and was not affected by ileitis ($P = 0.695$). Whatever the day of sampling and group, the PDV and dSI uptakes of arterial TCys were not different from zero. Although non-dietary cysteine release across the PDV and the dSI was numerically greater in the TNBS group, there was no interaction between day and group effects ($P > 0.10$), evidencing the lack of effect of ileitis on this parameter. Whatever the tracer used for calculation, TCys whole body flux was increased by ileitis induction. A schematic model of the different fluxes across the PDV is depicted in Fig. 4.

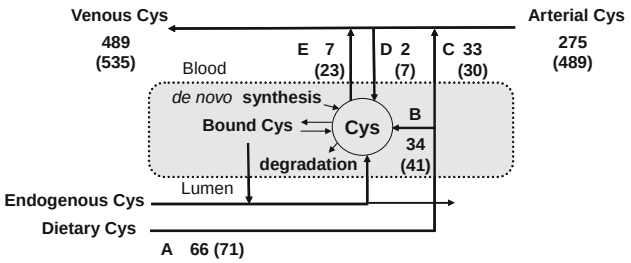

Fig. 4 Schematic representation of the metabolic fate of enteral and systemic cysteine in the portal-drained viscera of adult minipigs receiving intragastric infusion of an elemental solution. Values within brackets are for minipigs with TNBS-induced ileitis. *A* Intragastric infusion of cysteine. *B* First-pass uptake of dietary cysteine. *C* Release of dietary cysteine in the portal vein. *D* Uptake of arterial cysteine. *E* Release of non-dietary cysteine in the portal vein. Values are means (expressed as μmol kg^{-1} h^{-1}), $n = 8$

Glutathione concentration in the ileum was not affected ($P > 0.10$) by ileitis either in the healthy segment (1.6 ± 0.2 μmol/g) or in the inflamed one (1.9 ± 0.2 μmol/g). However, it tended to be greater ($P = 0.081$) in the liver of the TNBS group (6.5 ± 0.6 μmol/g) than in the Ctl group (5.2 ± 0.2 μmol/g).

[^{15}N] and [3,3D2] TCys isotopic enrichment in blood and tissues are presented in Table 2. Unfortunately, because of interferences due to sample matrix, it was not possible to reliably determine ^{15}N cysteine isotopic enrichment in the intestinal mucosa. Although ^{15}N and ^{2}H cysteine isotopic enrichments were not different in the portal vein, cysteine enrichment in the liver was threefold greater for ^{15}N than for ^{2}H. Similarly, cysteine isotopic enrichment in hepatic GSH was greater for ^{15}N than for ^{2}H. This indicates a preferential hepatic first-pass extraction of the dietary cysteine with respect to the circulating cysteine, which might be explained by the different forms of

Table 2 [^{15}N] and [D2]cysteine isotopic enrichment (molar percent excess) of cysteine (TCys) and glutathione (GSH), in tissues of control and TNBS-treated minipigs

	Control		TNBS		*t* test[a]		
	^{15}N-Cys	D2-Cys	^{15}N-Cys	D2-Cys	P_t	P_{t-15N}	P_{t-D2}
Aorta							
TCys	2.22 ± 0.05	2.84 ± 0.14	1.50 ± 0.14	2.12 ± 0.14	0.001	0.002	0.011
GSH	<0.2	<0.2	<0.2	<0.2	–	–	–
Portal vein							
TCys	2.45 ± 0.11	2.47 ± 0.09	1.61 ± 0.13	1.90 ± 0.13	0.201	0.002	0.012
GSH	<0.2	<0.2	<0.2	<0.2	–	–	–
Liver							
TCys	1.00 ± 0.10	0.31 ± 0.05	0.92 ± 0.04	0.29 ± 0.03	0.001	0.489	0.578
GSH	1.19 ± 0.14	0.98 ± 0.10	1.27 ± 0.06	0.91 ± 0.06	0.003	0.605	0.546
Proximal ileum							
TCys	–	0.87 ± 0.06	–	0.90 ± 0.10	–	–	0.861
GSH	1.00 ± 0.03	1.29 ± 0.07	0.81 ± 0.07	1.14 ± 0.04	0.001	0.129	0.113
Distal ileum							
TCys	–	0.82 ± 0.07	–	0.87 ± 0.06	–	–	0.582
GSH	0.65 ± 0.04	1.11 ± 0.07	0.72 ± 0.05	1.10 ± 0.05	0.001	0.330	0.851

Values are means ± SEM, $n = 4$

[a] P_t, probability for differences between tracers using paired *t* test, $n = 8$. $P_{t\ -\ 15N}$, probability for a treatment effect on [^{15}N]cysteine isotopic enrichment, unpaired *t* test, $n = 4$. $P_{t-D2,}$ probability for a treatment effect on [D2]cysteine isotopic enrichment, unpaired *t* test, $n = 4$

circulating labeled cysteine. Indeed, intravenous tracer probably bound rapidly to plasma proteins, whereas intragastric tracer released in the portal vein was largely in the form of cystine, which could be more available for tissues than protein-bound cysteine. For both liver and intestine, Ei TCys was lower than Ei GSH. This suggests either a channeling of extra cellular cysteine to GSH synthesis with low mixing with intracellular cysteine, or a low intracellular exchange of Cys with CysSSP. In both cases, TCys enrichment would not correspond to the precursor pool enrichment. Basically, for FSR calculations, the enrichment in a peptide/protein cannot overtake the enrichment in its precursor pool. As a consequence, the GSH FSRs calculated from the enrichment of the tissue TCys pool were erroneous (up to 1,500% day^{-1}). Nevertheless, the GSH FSRs were estimated using blood TCys as precursor pools, in combination with the intravenous or intragastric tracers (Table 3). Whatever the tracer and the precursor pool used in calculation, a trend ($P < 0.10$) or a significant increase ($P < 0.05$) in GSH FSR was observed in response to the TNBS challenge, both in the liver and the ileum.

Discussion

Cysteine was classically considered as a nutritionally dispensable amino acid. However, in certain pathological conditions associated with inflammation, it has been demonstrated to become indispensable since its endogenous synthesis is insufficient to cover the increased requirement (Breuillé and Obled 2000). During the acute phase of inflammation, the demand for this amino acid is increased to ensure the synthesis of the acute phase proteins (Malmezat et al. 1998) but also the synthesis of glutathione, the major endogenous antioxidant of the body, which is strongly stimulated in case of injury, infection and oxidative stress (Malmezat et al. 2000). Oral supplementation with cysteine in this condition was shown to be efficient to reduce nitrogen loss and muscle wasting, and finally to improve recovery in septic rats (Breuillé et al. 2006). However, this positive effect was only observed with a high level of oral cysteine (11 g/kg diet; cysteine accounting for 8% of the dietary AA), about three times the rat requirements (NRC 1995), raising the question of the oral cysteine bioavailability.

Cysteine trafficking across the PDV

Cysteine in plasma is predominantly in the form of CySS and portal net fluxes of cysteine previously reported in the literature are essentially CySS net fluxes. Based on CySS measurements, net portal recovery of dietary cysteine is low and ranged from 17% in piglets (Bos et al. 2003) to 50% in growing pigs (Rérat et al. 1992). It is about 45% in adult minipigs (Rémond et al. 2009). Additionally, it was shown that portal net flux of CySS accounts for only 4–28% of the cysteine that apparently disappeared from the small intestine of ruminants (Berthiaume et al. 2001; Rémond et al. 2003). Arteriovenous differences of TCys across the splanchnic bed were first investigated, in rats, by Garcia and Stipanuk (1992). However, since portal blood flows were not recorded, net fluxes were only an estimation. The present study is the first one reporting quantitative measurements of the portal net release of TCys across the PDV. This net flux accounted for about 60% of the dietary cysteine supply, suggesting a sequestration of about 40% of the dietary cysteine by the gut. This value, which is somewhat greater than the ones previously reported using

Table 3 Effect of ileitis on glutathione fractional synthesis rate (FSR, % day^{-1}) in enterally fed minipigs

	Control		TNBS-ileitis		*t* test[a]		
	^{15}N-Cys	D2-Cys	^{15}N-Cys	D2-Cys	P_l	P_{t-15N}	P_{t-D2}
Liver							
Artery	267 ± 45	153 ± 16	410 ± 50	192 ± 12	0.004	0.076	0.095
Portal vein	238 ± 35	174 ± 19	366 ± 36	219 ± 11	0.008	0.043	0.085
Proximal ileum							
Artery	194 ± 10	200 ± 7	260 ± 29	241 ± 3	0.710	0.078	0.001
Mesenteric vein	161 ± 7	229 ± 21	187 ± 7	287 ± 8	0.001	0.066	0.045
Distal ileum							
Artery	133 ± 9	173 ± 11	230 ± 24	232 ± 14	0.087	0.009	0.016
Mesenteric vein	105 ± 12	189 ± 12	171 ± 24	276 ± 19	0.001	0.082	0.018

FSR were estimated using different cysteine precursors for calculation

Values are means ± SEM, $n = 4$

[a] P_l, probability for differences between tracers using paired *t* test, $n = 8$. P_{t-15N}, probability for a treatment effect, using [^{15}N]cysteine in FSR calculation, unpaired *t* test, $n = 4$. P_{t-D2}, probability for a treatment effect, with [D2]cysteine in calculation, unpaired *t* test, n = 4

CySS measurements, suggested a significant contribution of non-CySS forms to TCys net release across the PDV.

The net flux only represents the balance between tissue uptake and release of the nutrient. Using dual intravenous and intragastric infusion of isotopically labeled cysteine, it was possible in the present study to distinguish these unidirectional fluxes. The approach thus allowed splitting of PDV TCys release according to the origin of cysteine (dietary vs. non-dietary), and it showed that about 60% of the dietary cysteine was actually sequestrated, at first pass, by the intestine. There is a substantial release of non-dietary cysteine by PDV tissues (15–25% of the TCys release in healthy minipigs). This cysteine could originate from endogenous production, by protein turnover or methionine metabolism, but also from reabsorption of the cysteine of the endogenous secretions (biliary GSH excretion, digestive enzymes, mucins, intestinal desquamation, etc.). Methionine transmethylation and transsulfuration is very active in the gastrointestinal tract, representing about a quarter of their respective whole body flux (Riedijk et al. 2007), but the non-dietary cysteine release by PDV tissues could also be explained by biliary GSH recycling. Large amounts of GSH are excreted with bile. In rats GSH biliary excretion was reported to be about 10 μmol kg^{-1} h^{-1} (Lauterburg et al. 1984). Part of this GSH could be reabsorbed intact (Hagen et al. 1990), via specific GSH transporters (Iantomasi et al. 1997), but it can also cross the enterocyte brush-border through the glutamyltranspeptidase, leading to intracellular production of free cysteine (Cys + CySS) that can be released into the blood stream. The quantitative significance of the endogenous release of cysteine by the PDV observed in the present study is consistent with such enterohepatic cycling of cysteine.

Surprisingly, we observed that the PDV did not extract cysteine from the arterial supply. Owing to the large amount of cysteine removed in a first pass at the absorption site, it is not so surprising for the small intestine. But for the other tissues of the PDV (stomach, large intestine, spleen, pancreas), which do not benefit from a direct supply of dietary cysteine, it means that they have to cover their requirements of cysteine through an endogenous synthesis from methionine, or from cysteine-containing peptides, this fraction of circulating cysteine being not accounted for in TCys flux measurements. The role of methionine is in agreement with the significant uptake of arterial methionine by the PDV tissues (30% of methionine whole body flux) previously observed in piglets (Riedijk et al. 2007). In addition, although the first-pass extraction of methionine by the gastrointestinal tract is low (Riedijk et al. 2007), we previously observed a PDV net release of methionine accounting for only 35% of the dietary supply, indicating a large uptake of arterial methionine. Furthermore, methionine presented the lowest apparent recovery among indispensable amino acids. All together, these observations argued in favor of a prominent role of arterial methionine in the supply of intracellular cysteine in PDV tissues. In the present experiment, the [3,3D2] cysteine isotopic enrichment observed in the ileal mucosa (about 30% of the arterial one) seems at odds with the lack of arterial cysteine extraction by the distal small intestine. As discussed above, a rapid recycling of GSH-cysteine via biliary secretion could explain this mucosal cysteine enrichment, but the uptake of circulating peptide-bound labeled cysteine could also be involved.

Impact of ileitis on cysteine fluxes

Whatever the tracer used in calculation (intravenous or intragastric one), the whole body cysteine flux was significantly increased by ileitis. The increase in the whole body flux of amino acids in pathological conditions associated with systemic inflammatory states is well documented (Mansoor et al. 1997; Obled et al. 2002). The specific evolution of cysteine whole body fluxes has been less well described. It was shown to increase in pediatric patients exhibiting sepsis (Lyons et al. 2001). In the present study, the inflammation was locally applied by administration of TNBS in the ileum. In this context, a 30–40% increase of cysteine flux appears of huge amplitude when related to such a local inflammation. This suggests that even an acute inflammation limited to a small surface of the intestine may increase cysteine utilization at the whole body level and thus impact on cysteine requirement. Although ileitis did not affect cysteine uptake by the gastrointestinal tract, it probably increased liver uptake of cysteine, in order to cover increased cysteine requirement for GSH and acute phase protein synthesis as already reported in other pathological conditions (Malmezat et al. 1998, 2000). In steady state conditions, such as in the present experiment, the rate of cysteine disappearance from blood compartment is equal to the rate of appearance, which therefore also increased in inflamed minipigs. Dietary cysteine release in the portal vein being not affected by ileitis, the increase in the rate of cysteine appearance into the blood compartment can only be explained by an increase in sulfur amino acid release through proteolysis and/or an increase in methionine transsulfuration. Indeed, an increase in transsulfuration was observed in humans submitted to a middle inflammation induced by vaccination (Mercier et al. 2006). The numeric increase in TCys net flux and endogenous TCys release across the PDV observed in the present study suggested that an increase in proteolysis and/or transsulfuration could also occur at the gastrointestinal tract level, during the acute phase of the inflammation.

In agreement with a previous study in septic rats (Malmezat et al. 2000), small intestinal GSH concentration

in the present study was not affected in the acute phase of the inflammation. However, ileitis induced a local increase of the FSR of GSH. As GSH concentration was not affected, it implies an increase in GSH consumption, probably in response to the local oxidative stress. Neither arterial nor luminal TCys uptake by the digestive tract was affected by the inflammation, and we previous reported no change in methionine portal net flux (Rémond et al. 2009). Thus, the increase in cysteine demand for GSH synthesis in the ileum was necessarily covered by an adaptation of the intensities of the various metabolic pathways of cysteine. It could be related to a decrease in cysteine oxidation as demonstrated at the whole body level during sepsis in rats (Malmezat et al. 2000). However, it could be also related to other adaptations of sulfur amino acid metabolism by decreasing the taurine synthesis from cysteine, or by increasing cysteine synthesis from methionine, or finally by the increased proteolysis. Further studies would be necessary to study this question.

In conclusion, this study provides, for the first time, in vivo measurement of unidirectional fluxes of TCys across the gastrointestinal tract. It shows that 60% of the dietary cysteine is retained in the gastrointestinal tract during absorption. Such observations strengthen the interest for cysteine derivatives escaping enterocyte metabolism during absorption, in order to increase cysteine delivery to the liver, which is the main contributor of whole body GSH synthesis in the acute phase of inflammation (Malmezat et al. 2000). Furthermore, this study indicates the absence of an arterial cysteine uptake by the gastrointestinal tract, at least in molecular forms that do not involve peptide bonds. This suggests a preferential use of methionine or cysteine-containing peptides in order to cover cysteine requirement of the PDV that do not benefit from a direct digestive supply. A significant part of the demand for cysteine in the gastrointestinal tract is driven by GSH synthesis, but also mucin synthesis (cysteine-rich protein). During the acute phase of inflammation, the increase in mucin synthesis is actually accompanied by a large increase in threonine uptake by the gastrointestinal tract, in agreement with the high amount of threonine in mucins (Rémond et al. 2009). In the present work, it was shown that despite the increase in mucin and GSH synthesis, acute ileitis does not impact on first-pass sequestration of dietary cysteine and on arterial cysteine uptake by the PDV, suggesting an intracellular reorientation of sulfur amino acid metabolism. This study also evidences the need for a better knowledge of the quantitative significance of cysteine enterohepatic recycling, and of cysteine di- and tripeptide involvement in cysteine interorgan fluxes.

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Appendix

Cysteine flux calculations

- Whole body cysteine flux (Ra, μmol kg^{-1} h^{-1}) was derived from the equation

$$Ra = Inf \times ((Ei\ Cys_{inf}/Ei\ TCys_{art}) - 1)$$

where Inf is the rate of infusion of tracer (μmol kg^{-1} h^{-1}), Ei Cys_{inf} and Ei $TCys_{art}$ are isotopic enrichments (MPE) in infused solution and arterial blood, respectively. Ra was calculated with intravenous tracer (Ra_{iv}) or intragastric tracer (Ra_{ig}).

- Arterial blood flow to the PDV (BF_a) was estimated as follows:

$$BF_a = BF_v \times Hb_v/Hb_a$$

where BF_v is measured blood flow (L kg^{-1} h^{-1}) in the portal vein, and Hb_a and Hb_v are hemoglobin concentrations (g/L) in arterial and venous whole blood, respectively. Hemoglobin ratio was used in order to take into account water movement across the portal-drained viscera.

- Similarly venous blood flow from the dSI was estimated as:

$$BF_v = BF_a \times Hb_a/Hb_v.$$

- PDV and dSI TCys net fluxes (F_{net} TCys, μmol kg^{-1} h^{-1}) were calculated as:

$$F_{net}TCys = \left([TCys]_v \times BF_v\right) - \left([TCys]_a \times BF_a\right)$$

where [TCys] is the blood cysteine concentration (μmol/L), BF is blood flow, and subscripts a and v refer to artery venous data, respectively.

- PDV and dSI extraction rates of arterial TCys (Ext $TCys_{art}$, %) were calculated from the equation

$$Extr\,TCys_{art} = \left(\left([TCys]_a \times Ei\,TCys_a\right) - \left([TCys]_v \times Ei\,TCys_v\right)\right)/\left([TCys]_a \times Ei\,TCys_a\right) \times 100$$

where Ei TCys is isotopic enrichment (MPE) of intravenous tracer in the artery and vein, respectively.

- PDV and dSI uptake of arterial TCys (Capt $TCys_{art}$, μmol kg^{-1} h^{-1}) was calculated from the equation

$$Capt\ TCys_{art} = [TCys]_a \times BF_a \times Extr\ TCys_{art}.$$

- First-pass appearance of intragastric tracer in portal blood (Ap $TCys_{lum}$, μmol kg^{-1} h^{-1}) was calculated from the equation

$$\begin{aligned}\text{Ap TCys}_{\text{lum}} = &\left(\left([\text{TCys}]_{\text{v}} \times \text{Ei TCys}_{\text{v}} \times \text{BF}_{\text{v}}\right)\right.\\ &- \left([\text{TCys}]_{\text{a}} \times \text{Ei TCys}_{\text{a}} \times \text{BF}_{\text{a}}\right)\\ &+ \left([\text{TCys}]_{\text{a}} \times \text{Ei TCys}_{\text{a}} \times \text{Extr TCys}_{\text{art}} \times \text{BF}_{\text{a}}\right)\end{aligned}$$

in which Ei TCys_{art} et Ei TCys_{v} are isotopic enrichments (MPE) of intragastric tracer in the artery and vein, respectively.

– First-pass extraction rate of intragastric tracer by the PDV ($\text{Ext}_{\text{portal}}$, %) was

$$\text{Ext}_{\text{portal}} = (\text{Inf TCys}_{\text{lum}} - \text{Ap TCys}_{\text{lum}})/(\text{Inf TCys}_{\text{lum}}) \times 100$$

where Inf TCys_{lum} is the flux of tracer perfused in the stomach (μmol kg^{-1} min^{-1}). It was assumed that the entire intragastric tracer was absorbed from the intestine.

– First-pass utilization of dietary cysteine by the PDV (Capt TCys_{lum}, μmol kg^{-1} h^{-1}) was

$$\text{Capt TCys}_{\text{lum}} = \text{Ext}_{\text{portal}}\text{TCys}_{\text{lum}} \times \text{dietary TCys intake}.$$

– Portal release of cysteine not derived from the diet (Ap $\text{TCys}_{\text{endo}}$, μmol kg^{-1} h^{-1}) was calculated as follows

$$\begin{aligned}\text{Ap TCys}_{\text{endo}} = &\text{F}_{\text{net}}\ \text{TCys}\\ &- (\text{dietary TCys intake} - \text{Capt TCys}_{\text{lum}})\\ &+ \text{Capt TCys}_{\text{art}}.\end{aligned}$$

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