Nutrient digestion and absorption during chemotherapy-induced intestinal muscositis in the rat

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CHAPTER 5

CONTINUOUS ENTERAL ADMINISTRATION CAN ENABLE NORMAL AMINO ACID ABSORPTION IN RATS WITH METHOTREXATE-INDUCED GASTROINTESTINAL MUCOSITIS

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ABSTRACT

Background It is unknown what feeding strategy to use during chemotherapy-induced gastrointestinal mucositis, causing weight loss and possibly malabsorption. We aimed to study the absorptive capacity of amino acids during mucositis. **Methods** We determined the plasma availability of enterally administered amino acids (AA), their utilization for protein synthesis, and the preferential side of the intestine for AA uptake in rats with and without methotrexate (MTX)-induced mucositis. Four days after injection with MTX (60 mg/kg) or saline (controls), rats received a primed, continuous dual-isotope infusion (intraduodenal and intravenous) of labeled L-leucine, L-lysine, L-phenylalanine, L-threonine and L-methionine. We took blood samples, assessed jejunal histology and determined labeled AA incorporation in proximal and distal small intestinal mucosa, plasma albumin, liver and thigh muscle. **Results** MTX-induced mucositis was confirmed by histology. The median systemic availability of all AA except for leucine was similar in MTX-treated rats and in controls. However, individual availability of all AA differed substantially within the group of MTX-treated rats, ranging from severely reduced (<10% of intake) to not different from controls (>40% of intake in 5 of 9 rats). More AA originating from basolateral uptake than those originating from apical uptake were used for intestinal protein synthesis in MTX-treated rats (at least 420% more, $P<0.05$). **Conclusions** We conclude that continuous enteral administration can enable normal AA absorption in rats with MTX-induced mucositis. The intestine prefers basolateral AA uptake to meet its need for AA for protein synthesis during mucositis.

INTRODUCTION

Gastrointestinal mucositis (further referred to as “mucositis”) is one of the most severe and debilitating side effects of anti-cancer treatment, causing small intestinal villus atrophy and loss of enterocytes [1]. Patients with mucositis suffer from anorexia, nausea, diarrhea and weight loss [2]. It is unknown how to optimally feed patients with mucositis, although nutritional support might improve the nutritional state, accelerate recuperation and increase survival of mucositis patients [3-6]. Normally, enteral nutrition, which is the physiological way of feeding, is preferred to total parenteral nutrition (TPN) because the latter carries a high risk of infection and, upon prolonged administration, may cause liver disease [7, 8]. However, when the absorptive function of the intestine is compromised, TPN offers a useful feeding alternative.

We developed a methotrexate (MTX)-induced mucositis rat model to determine nutrient digestion and absorption during mucositis, and to ultimately design a rational feeding strategy for mucositis patients [9]. In this model, we showed that trace amounts of glucose are absorbed normally during mucositis [9]. Because there are

indications that intestinal absorption of amino acids (AA) might be intact during mucositis [10], in contrast to absorption of di- and tripeptides [11], we here aimed to determine the capacity to absorb enterally administered AA during mucositis.

AA serve several important functions in the human body [12], particularly during periods of growth [13], and play an important role in mucosal homeostasis [14, 15]. Normally, the intestine itself metabolizes a substantial part (up to 80%) of nutrients after absorption from the intestinal lumen before nutrients become systemically available; a process called 'first-pass splanchnic utilization' [12, 16-19]. When more nutrients are used for first-pass utilization, fewer nutrients are systemically available for whole-body energy metabolism and peripheral tissue synthesis [12]. A unique feature of intestinal enterocytes is that they do not only absorb AA directly from the lumen by their apical membrane, they can also take up AA from the mesenteric arterial circulation by their basolateral membrane after becoming systemically available [12, 20-22]. It is not well known how mucositis affects the first pass splanchnic uptake and the resulting systemic availability of AA, or to what extent systemically available AA are used for protein synthesis in diverse tissues. Knowledge about these processes is needed in order to determine the absorptive capacity of enterally administered AA, and whether the gut can be used for uptake of AA during mucositis. Furthermore, we hypothesized that there could be a preferential side of the intestine for AA uptake, in order to synthesize proteins during mucositis.

To study the absorptive capacity of amino acids during mucositis, we determined the plasma availability of five enterally administered, essential AA (to indirectly test the function of different AA transporter systems), their utilization for protein synthesis, and the preferential side of the intestine for AA uptake in rats with and without MTX-induced mucositis. We determined absorption of a physiologically relevant amount of AA (i.e. a normal hourly AA intake, instead of a trace amount of AA) when continuously administered by intraduodenal (i.d.) infusion, since continuous enteral nutrient administration has been shown to improve absorption of another nutrient during mucositis in the rat; i.e. glucose [23].

MATERIALS AND METHODS

Rats and housing

Male Wistar outbred rats (4 wk old, 65-75 g, Specific Pathogen Free) were obtained from Charles River (Sulzfeld, Germany). Rats were individually housed in Plexiglas cages (42.5 x 26.6 x 18.5 cm) on a layer of wood shavings under controlled temperature (21 $\pm$ 1 °C) with a relative humidity of 55 $\pm$ 10% and a 12 h light - 12 h dark cycle (lights on 07.00-19.00 h). Water and purified diet (AIN-93G [24], Research Diet Services B.V., Wijk bij Duurstede, the Netherlands) were available ad libitum unless otherwise stated. The experimental protocol was approved by the Ethics

Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

Materials

MTX was obtained from Pharmachemie Holding B.V. Stable isotope-labeled AA of 88-99% isotopic purity for i.d. and intravenous (i.v.) infusion (**Table 1**) were purchased from CortecNet. Unlabeled AA for i.d. infusion (Table 1) were purchased from Sigma-Aldrich Chemie GmbH.

	Infusion rate, $\mu mol \cdot kg^{-1} \cdot h^{-1}$
Intraduodenal	
Stable isotope-labeled	
L-[1,2-$^{13}C_2$]leucine	117
L-[$^{13}C_6$, $^{15}N_2$]lysine-HCl	107
L-[1-^{13}C]phenylalanine	73
L-[^{13}C4, ^{15}N]threonine	46
L-[$^{13}C_5$, ^{15}N]methionine	26
Unlabeled	
L-leucine	262
L-lysine-HCl	218
L- phenylalanine	86
L- threonine	153
L-methionine	68
Intravenous	
Stable isotope-labeled	
L-[5,5,5-$^{2}H_2$]leucine	228
L-[$^{15}N_2$]lysine-HCl	181
L-[$^{2}H_5$]phenylalanine	75
L-[^{15}N]threonine	146
L-[$^{2}H_3$]methionine	56

Table 1. Rates of intraduodenal and intravenous infusion of stable isotope-labeled and unlabeled essential amino acids in control and MTX-treated rats. *Values are absolute. For individual rats, values were adjusted to their individual body weight on d 4 (range 185-250 g), n = 16. MTX, methotrexate.*

Experimental procedures

AA infusion protocol in the mucositis rat model. One week after arrival at the animal facility, rats were equipped with permanent catheters in the duodenum and jugular vein as described previously [25]. One week after surgery, rats (6 wk old, 205-250 g) were injected once i.v. in the tail vein with MTX (60 mg/kg, n=9) to induce mucositis or with saline (0.9%, controls, n=7) under general anesthesia [9]. Assignment of rats to one of the treatment groups was randomly performed by the researcher (MF). Intake of food and water, body weight and the presence of diarrhea [present as watery diarrhea or absent [9]] were recorded daily at ~08.00 h. Four days after injection, when histological and clinical symptoms of MTX-induced mucositis were most severe [9], the AA absorption experiment was performed. A primed (once the hourly dose), continuous i.d. and i.v. infusion of AA in distilled water was started in unanesthetized rats for 5 h after an overnight feed deprivation (23.00 h on d 3 to 08.00 h on d 4) to reach a steady state [26]. Infusion rates and doses of the i.d. and i.v. infusates (Table 1) were based on results from pilot studies that we had executed earlier (unpublished material). Unlabeled AA were added to stable isotope-labeled AA in the i.d. infusate to reach a normal intake of each amino acid during the experimental period, based on the mean daily AA intake in

control rats (i.e. 5/24 x mean daily AA intake in controls, calculated from their mean daily food intake which is ±20g AIN93G per 230g body weight [9]), to study physiological AA absorption instead of studying a tracer effect. Blood samples were obtained at baseline and in steady state (at 4, 4.5 and 5 h after the start of the dual-isotope infusion, based on pilot studies and as done previously [26]) for mass spectrometry (MS) analyses. After the 5-h AA infusion protocol, rats were killed under general anesthesia by obtaining a large blood sample through cardiac puncture for determination of plasma albumin and citrulline concentrations, followed by cervical dislocation. Blood samples were centrifuged immediately (10 min at 2,000 x *g*) and collected plasma was stored at -80°C until further analysis.

Tissue collection. Immediately after rats were killed, the abdomen was opened via a midline incision and the small intestine, liver and a sample from the thigh muscle were quickly removed. After the small intestine was flushed with ice-cold PBS, a small part of the jejunum (anatomical middle of the small intestine) was collected for histology and fixed in formalin (1 cm) or 2% paraformaldehyde (PFA, 1 cm) dissolved in PBS, dehydrated and embedded in paraffin according to standard procedures for histochemistry [9]. Mucosa of the rest of the small intestine, being a proximal part (between stomach and anatomical middle, i.e. duodenum and proximal jejunum) and a distal part (between anatomical middle and cecum, i.e. distal jejunum and ileum), was scraped on ice, weighed (wet weights) and stored at -80°C until further analysis. Liver and thigh muscle were weighed (wet weights), freeze-clamped, pulverized in liquid nitrogen and stored at -80°C until further analysis.

Analytical methods

Hematoxylin-and-eosin (H&E) staining of formalin- and paraformaldehyde-fixed jejunal segments to assess histology, as well as their morphometric analysis, was carried out as described previously [9]. Plasma citrulline concentrations [indicating functional enterocyte mass [27]] were measured as described previously [9]. Plasma albumin concentrations were measured in 150 µL plasma via the bromocresol green method, which is a calorimetric assay, as described by the manufacturer (Roche Diagnostics GmbH). Tissue samples (± 50 mg) of proximal and distal small intestinal mucosa, liver and thigh muscle were homogenized in distilled water (± 500 µL) to measure tissue isotopic enrichment of all AA (see 'Tissue enrichment analyses' below). An aliquot of 50 µL was taken to measure tissue protein concentrations according to Lowry et al. [28].

Mass spectrometry

Plasma enrichment analysis. Plasma samples (30 µL) were prepared to determine isotopic enrichment of all AA by gas chromatography-MS (MSD 5975C, Agilent Technologies), as described previously [26, 29-31]. Instead of plasma leucine enrichment, plasma enrichment of its keto-analogue α-ketoisocaproic acid was

measured to correct for intracellular transamination of leucine, as done before by us and others [10, 32]. The enrichment of all AA in steady state was calculated by using the mean enrichment between 4 and 5 h after continuous dual-isotope infusion, corrected for the enrichment at baseline, as described previously [33]. Enrichment was expressed in mole percent excess.

Tissue enrichment analysis. Aliquots of 200 µL homogenized tissue (see 'Analytical methods' above) were taken to measure isotopic enrichment of all free (unbound) and protein-bound AA. The protein fraction was isolated and analyzed as previously described [30]. In short, proteins were precipitated and the supernatant was collected and used for enrichment analysis of free AA. The washed, precipitated protein pellets were hydrolyzed by adding 1 mL of 6 mol/L HCl and incubated at 110°C for 20 h. An aliquot was dried at room temperature in a speedvac (GeneVac miVac, GeneVac Ltd), and the residue was dissolved in 0.2 mL milli-Q. AA were isolated by cation exchange separation. To measure the enrichment of AA in the protein-bound tissue pool, hydrolyzed samples were derivatized to form acetyl-ethoxycarbonyl-ethylesters. The ^{13}C:^{12}C ratio of AA in protein isolates were measured by using a gas chromatograph/combustion/isotope ratio MS (Delta XP, Thermo Fisher) according to the method used in previous work [26, 31]. Isotopic enrichment of protein-bound ^{2}H- or ^{15}N-labeled AA, and of all free AA in the supernatants, was determined by gas chromatography/MS analysis of their acetyl-ethoxycarbonyl-ethylesters by using electron impact ionization, as described for the plasma samples. Enrichment was expressed in mole percent excess.

Plasma albumin analysis. Albumin was isolated from collected plasma at 4, 4.5 and 5 h after the start of the dual-isotopically labeled AA infusions. After hydrolysis, the isotopic enrichment of enterally administered AA was measured by using a gas chromatograph/combustion/isotope ratio MS to determine the synthesis rate of plasma albumin as described previously [34, 35].

Values obtained for isotopic enrichment of all AA (including α-ketoisocaproic acid) were corrected for the contribution of natural abundance on the measured fragments, as well as for the contribution of administered tracers to the measured fragments.

Calculations

The equations used to obtain the results are detailed in the Supplemental Methods.

Statistical analysis

Statistical analysis of data in MTX-treated rats versus controls (i.e. rat characteristics, AA kinetics, and protein synthesis) was performed by using the Mann-Whitney *U*-test (SPSS 16.0 for Windows, SPSS). Analysis of data on basolateral versus apical AA uptake for protein synthesis in MTX-treated rats or in controls (see 'Supplemental Methods')

was performed by using the Wilcoxon signed-rank test. Data are presented as absolute values (Table 1) and median and range (Tables 2-5) or as data for individual rats (Supplemental Figure 1) for the indicated number of rats (*n*) per group. Correlations are expressed as nonparametric Spearman correlation coefficient. For significant correlations, optimal curve fitting was performed by using non-linear regression with a polynomial model (Supplemental Figure 1). $P<0.05$ was considered significant.

RESULTS

The mucositis rat model

We studied the capacity to absorb enterally administered AA during mucositis in a previously established MTX-induced mucositis rat model [9]. As seen in previous studies by us and others [9, 36-41], MTX-treated rats showed typical histological and clinical symptoms of mucositis (i.e. villus atrophy, a reduced plasma citrulline concentration, a reduced intake, weight loss and watery diarrhea), in contrast to controls (**Table 2**). Although symptoms of mucositis varied from mild to severe in individual rats, most MTX-treated rats (7 of 9) suffered from severe mucositis [i.e. villus length <300 µm and plasma citrulline concentration <30 µmol/L [9]] (**Supplemental Figure 1**).

	Control	MTX
Villus length d 4, µm	408 (375-423)	262 (198-354) *
Citrulline d 4, µmol/L plasma	69 (54-105)	15 (10-55) *
Food intake d 3, gr/d	11 (8-12)	0 (0-9) *
Body weight d 4[1], %	108 (105-110)	91 (88-104) *
Diarrhea d 4[2], % of rats	0	67

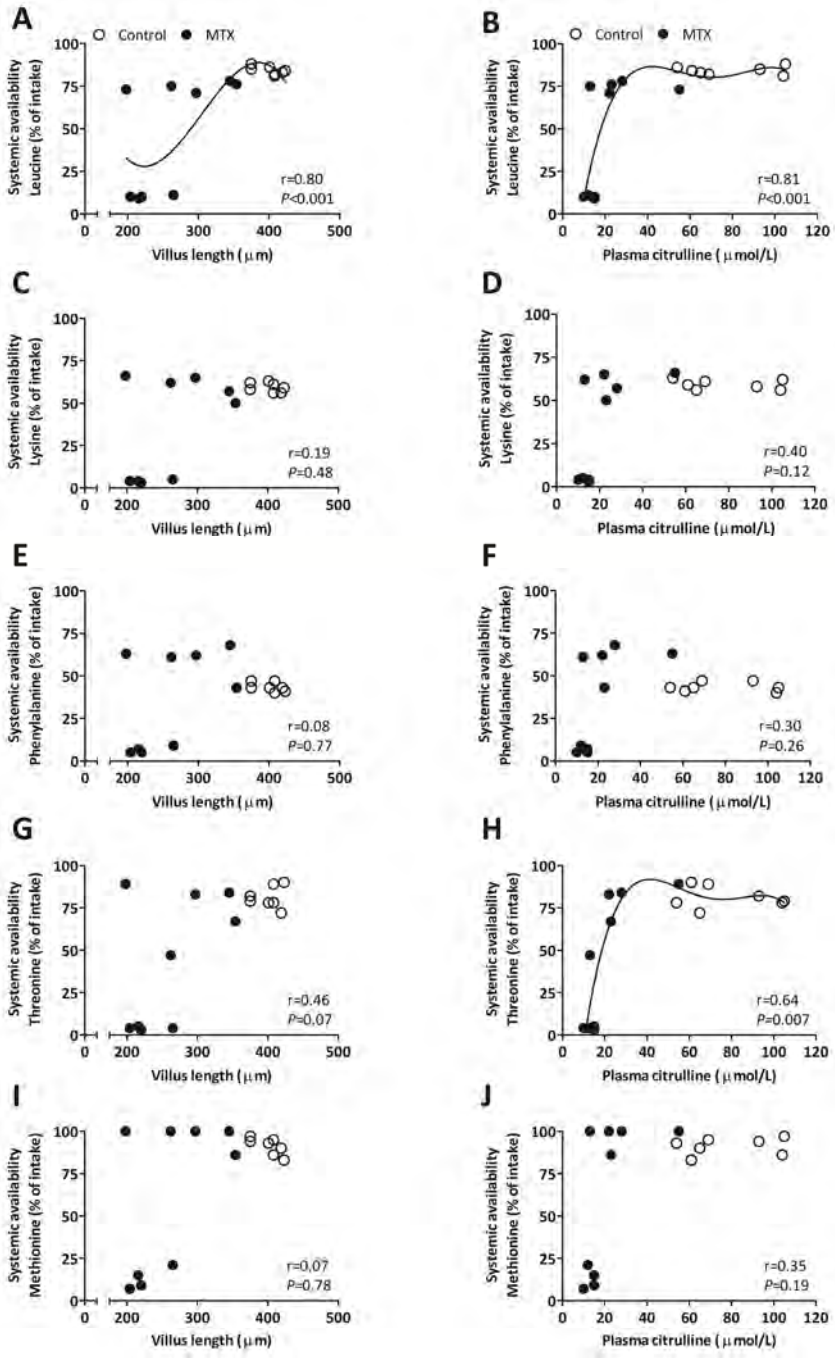
Table 2. Characteristics of control and MTX-treated rats. *Values are medians and range, except for diarrhea for which values are absolute, n = 7-9. *Different from control, P<0.01. MTX, methotrexate.*

[1] *Body weight was relative to weight at d 0 (day of MTX or saline injection), which was arbitrarily set at 100%.*

[2] *Diarrhea was present as watery diarrhea or completely absent.*

Plasma kinetics of i.v.- and i.d.-infused AA

As shown in **Table 3**, median plasma AA fluxes, based on i.v.-infused tracers and on i.d.-infused tracers, were similar in MTX-treated rats and in controls for 4 out of 5 AA. However, MTX-treated rats showed substantial interindividual differences in AA fluxes based on i.d.-infused tracers, with maximal values 500-1400% higher than maximal values in controls. This was due to a large variability in individual plasma enrichment of i.d.-infused tracers in MTX-treated rats (ranging from severely reduced to normal, data not shown).



Supplemental Figure 1. *Correlation between systemic availability of intraduodenally infused stable isotope-labeled amino acids on the one hand and villus length (A, C, E, G and I) or plasma citrulline concentration (B, D, F, H and J) on the other hand in control (○) and methotrexate (MTX)-treated (●) rats. Values are data of individual rats, n=7-9. Spearman correlations (r) and P values are indicated. For significant correlations (P<0.05), optimal curves were plotted using non-linear regression with a polynomial model (A, B and H).*

	Leucine		Lysine		Phenylalanine		Threonine		Methionine	
	Control	*MTX*	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*
Flux intravenous tracer, mmol·kg^{-1}·h^{-1}	0.95 (0.92-1.00)	0.92 (0.58-1.00)	0.63 (0.61-0.71)	0.58 (0.47-0.69)	0.35 (0.32-0.37)	0.35 (0.27-0.41)	0.58 (0.55-0.68)	0.51 (0.37-0.55)*	0.32 (0.31-0.36)	0.35 (0.26-0.39)
Flux intraduodenal tracer, mmol·kg^{-1}·h^{-1}	1.13 (1.10-1.15)	1.38 (1.20-7.98)*	1.09 (1.00-1.15)	1.16 (1.00-16.03)	0.83 (0.74-0.87)	0.76 (0.56-5.48)	0.72 (0.65-0.86)	1.08 (0.62-12.84)	0.36 (0.33-0.39)	0.35 (0.31-3.76)
First-pass splanchnic utilization, % of intake	16 (12-19)	29 (22-91)*	41 (37-44)	50 (34-97)	57 (53-60)	57 (32-95)	21 (10-28)	53 (11-97)	7 (3-17)	14 (0-93)
Systemic availability, % of intake	84 (81-88)	71 (9-78)*	59 (56-63)	50 (3-66)	43 (40-47)	43 (5-68)	79 (72-90)	47 (3-89)	93 (83-97)	86 (7-100)
Protein breakdown, mmol·kg^{-1}·h^{-1}	0.63 (0.60-0.67)	0.69 (0.55-0.80)*	0.43 (0.41-0.51)	0.46 (0.41-0.51)	0.29 (0.25-0.30)	0.27 (0.26-0.34)	0.42 (0.40-0.52)	0.38 (0.36-0.43)*	0.24 (0.23-0.27)	0.26 (0.22-0.33)*

Table 3. Kinetics of intravenously- and/or intraduodenally infused, stable isotope-labeled amino acids in control and MTX-treated rats. Values are medians and range, n=7-9.
*Different from control, P<0.05. MTX, methotrexate.

Median first-pass splanchnic utilization, and resulting systemic availability, of all i.d.-infused AA except for leucine were not different between MTX-treated rats and controls. In contrast to controls, MTX-treated rats showed substantial inter-individual differences (Table 3). Maximal first-pass splanchnic utilization of all AA was >90% of intake in MTX-treated rats, whereas it was ≤60% of intake in controls. As a result, minimal systemic availability of all AA was <10% of intake in MTX-treated rats, while it was ≥40% of intake in controls. Availability of all AA varied from severely reduced (<10% of intake) to not different from controls among individual MTX-treated rats with symptoms of severe mucositis (7 of 9 rats, Supplemental Figure 1). Although the systemic availability of enterally administered leucine in all rats correlated with villus length (r=0.80, *P*<0.05), that of lysine, phenylalanine, threonine and methionine did not. Similarly, the systemic availability of enterally administered leucine and threonine in all rats correlated with plasma citrulline (r=0.64-0.81, *P*<0.05) but that of lysine, phenylalanine and methionine did not.

Protein breakdown in MTX-treated rats, based on i.d.-infused tracers, varied according to AA and was higher than (leucine and

methionine, *P*<0.05), lower than (threonine, *P*<0.05) or similar to controls (lysine and phenylalanine, Table 3).

Tissue protein and albumin synthesis with i.d.-infused AA

The utilization of enterally administered AA for protein synthesis was expressed by the fractional and absolute synthesis rate of protein (FSR and ASR respectively), indicating the relative and absolute need for AA for protein synthesis, respectively.

Small intestinal mucosa. The FSR with systemically available, enterally administered AA ($FSR_{basolateral}$) was ≥ 20% higher in MTX-treated rats than in controls, depending on the specific AA (proximal and distal mucosa, P<0.05, **Table 4**). However, since the total amounts of mucosa (proximal and distal mucosa ≥49% lower, *P*<0.05) and/or the protein concentration of mucosa (proximal mucosa 16% lower, *P*<0.05) were lower in MTX-treated rats than in controls, the ASR ($ASR_{basolateral}$) was lower in MTX-treated rats than in controls (proximal mucosa, *P*<0.05) or similar to controls (distal mucosa). In both MTX-treated rats and in controls, protein synthesis in proximal and distal small intestinal mucosa was higher with enterally administered AA taken up from the systemic side ($FSR_{basolateral}$ and $ASR_{basolateral}$) than with AA taken up from the luminal side (FSR_{apical} and ASR_{apical}, *P*<0.05). These differences between basolateral and apical AA uptake seemed to be more pronounced for MTX-treated rats than for controls, both in proximal and distal mucosa. The enrichment of methionine was too low to measure.

Albumin. The FSR with systemically available, enterally administered AA was ≥60% higher in MTX-treated rats than in controls, depending on the specific AA (*P*<0.05, **Table 5**). However, since the plasma albumin concentration was lower in MTX-treated rats than in controls (24% lower, *P*<0.05), the ASR was similar in MTX-treated rats and in controls. The enrichment of threonine and methionine was too low to measure.

Liver. The FSR with systemically available, enterally administered AA was 20% and 30% higher in MTX-treated rats than in controls for leucine and lysine, respectively (*P*<0.05, Table 5), or similar to controls for phenylalanine. Both the total amounts of liver and the protein concentrations in liver were similar in MTX-treated rats and in controls, and therefore the ASR was also 20% and 30% higher in MTX-treated rats than in controls for leucine and lysine respectively (*P*<0.05), or similar to controls for phenylalanine. The enrichment of threonine and methionine was too low to measure.

Thigh muscle. The FSR with systemically available, enterally administered AA was similar in MTX-treated rats and in controls (Table 5). The protein concentration of thigh muscle was similar in MTX-treated rats and in controls, and therefore the ASR (per kg muscle) was also similar in both groups. The enrichment of threonine and methionine was too low to measure.

Table 4. Amount, protein concentration, FSR and ASR of small intestinal mucosa using intraduodenally infused, stable isotope-labeled amino acids in control and MTX-treated rats. *Values are medians and range, n=7-9. * Different from control, P<0.05. # Different from apical FSR or apical ASR, P<0.05. ASR, absolute synthesis rate; BW, body weight; FSR, fractional synthesis rate; MTX, methotrexate; ND, not detectable.*

[1] *FSR and ASR with intraduodenally infused amino acids via basolateral uptake (from the systemic side).*

[2] *FSR and ASR with intraduodenally infused amino acids via apical uptake (from the luminal side).*

			Leucine		Lysine		Phenylalanine		Threonine	
Proximal mucosa	**Control**	**MTX**	**Control**	**MTX**	**Control**	**MTX**	**Control**	**MTX**	**Control**	**MTX**
Total amount of mucosa, g	16.1 (8.7-17.2)	6.3 (2.0-7.7)*								
Mucosal protein concentration, g/kg	108 (99-141)	91 (71-111)*								
Total amount of mucosal protein, g/kg BW	7.6 (5.0-8.1)	2.4 (0.9-4.6)*								
$FSR_{basolateral}$ [1], %/d			77 (70-84)#	117 (63-155)* #	51 (48-56)#	89 (46-108)* #	59 (54-65)#	95 (54-130)* #	72 (69-79)#	84 (62-131)#
$ASR_{basolateral}$ [1], $g \cdot kg\ BW^{-1} \cdot d^{-1}$			5.5 (4.1-6.3)#	2.9 (0.9-4.5)* #	3.9 (2.7-4.4)#	1.9 (0.6-3.6)* #	4.3 (3.2-5.0)	2.1 (0.6-5.9)* #	5.4 (3.9-5.6)#	2.0 (0.8-5.3)* #
FSR_{apical} [2], %/d			19 (14-24)	4 (0-14)*	14 (10-22)	4 (0-11)*	47 (43-54)	16 (13-30)*	ND	ND
ASR_{apical} [2], $g \cdot kg\ BW^{-1} \cdot d^{-1}$			1.2 (0.9-1.7)	0.0 (0.0-0.3)*	1.1 (0.8-1.3)	0.0 (0.0-0.3)*	3.3 (2.2-4.4)	0.4 (0.1-0.7)*	ND	ND
Distal mucosa	**Control**	**MTX**	**Control**	**MTX**	**Control**	**MTX**	**Control**	**MTX**	**Control**	**MTX**
Total amount of mucosa, g	14.3 (11.1-19.3)	7.3 (3.0-13.4)*								
Mucosal protein concentration, g/kg	96 (65-110)	86 (62-107)								
Total amount of mucosal protein, g/kg BW	5.9 (3.8-7.9)	3.6 (1.2-4.9)*								
$FSR_{basolateral}$ [1], %/d			67 (40-80)#	103 (54-188)* #	54 (45-62)#	89 (45-121)* #	77 (69-89)#	99 (61-132)* #	56 (36-75)#	105 (50-148)* #
$ASR_{basolateral}$ [1], $g \cdot kg\ BW^{-1} \cdot d^{-1}$			3.8 (2.5-5.1)#	3.6 (1.6-7.5)#	3.0 (2.1-3.3)#	3.2 (1.2-5.0)#	4.6 (3.1-5.5)#	3.2 (1.1-5.5)#	3.1 (2.2-3.7)#	2.3 (1.3-6.2)#
FSR_{apical} [2], %/d			ND	0.0 (0.0-11.0)	0.0 (0.0-1.0)	0.4 (0.0-7.8)	11 (1-14)	19 (7-26)	ND	ND
ASR_{apical} [2], $g \cdot kg\ BW^{-1} \cdot d^{-1}$			ND	0.0 (0.0-0.4)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.5 (0.1-0.9)	0.4 (0.2-0.9)	ND	ND

			Leucine		Lysine		Phenylalanine	
Plasma albumin	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*
Albumin concentration, g/l plasma	37 (28-39)	28 (25-33)*						
FSR, %/d			25 (16-30)	49 (11-102)*	19 (15-27)	30 (14-70)*	60 (14-91)	112 (33-198)*
ASR, $g \cdot L^{-1} \cdot d^{-1}$			9.3 (4.6-11.2)	12.2 (3.5-25.5)	6.8 (4.1-10.0)	8.4 (4.5-17.4)	22.1 (5.2-33.6)	28.1 (10.6-49.6)
Liver	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*
Total amount of liver, g	8.9 (7.8-9.7)	8.2 (7.0-9.3)						
Liver protein concentration, g/kg	228 (208-240)	215 (194-235)						
Total amount of liver protein, g/kg BW	8.5 (7.7-9.0)	8.6 (8.2-10.5)						
FSR, %/d			42 (42-50)	51 (43-77)*	35 (28-39)	45 (31-76)*	86 (76-97)	84 (72-115)
ASR, $g \cdot kg\ BW^{-1} \cdot d^{-1}$			3.8 (3.4-4.2)	4.5 (3.6-7.1)*	2.9 (2.3-3.3)	3.9 (2.7-6.7)*	7.5 (6.0-8.2)	7.5 (6.2-10.5)
Thigh muscle	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*	*Control*	*MTX*
Muscle protein concentration[1], g/kg	42 (33-47)	45 (31-59)						
FSR, %/d			7.2 (4.5-9.5)	4.9 (0.8-9.5)	5.4 (2.9-7.6)	3.5 (0.8-6.1)	12.6 (7.4-18.1)	8.1 (5.0-15.7)
ASR, $g \cdot kg\ muscle^{-1} \cdot d^{-1}$			2.9 (2.0-3.7)	2.1 (0.3-4.4)	2.2 (1.3-3.0)	1.1 (0.4-3.6)	5.4 (3.2-7.1)	3.5 (1.8-7.2)

Table 5. Amount, protein concentration, FSR and ASR of albumin, liver and thigh muscle using intraduodenally infused, stable isotope-labeled amino acids in control and MTX-treated rats. * Different from control, P<0.05. ASR, absolute synthesis rate; BW, body weight; FSR, fractional synthesis rate; MTX, methotrexate.

[1] The total amount of muscle per rat is unknown; therefore, the total amount of muscle protein per kilogram BW cannot be calculated.

DISCUSSION

We aimed to determine the absorptive capacity of AA in rats with and without MTX-induced mucositis. Our data indicate that continuous enteral administration can enable normal AA absorption in rats with MTX-induced mucositis. The intestine prefers basolateral AA uptake to meet its need for AA for protein synthesis during mucositis.

The absorptive capacity of AA was determined in a previously established MTX-induced mucositis rat model [9]. We chose five essential AA that are absorbed by different transporter systems, normally present on the apical and basolateral membrane of intestinal enterocytes [42, 43], to indirectly test the function of all these systems during mucositis. In earlier studies, only the absorption of leucine was studied during mucositis in children [10]. AA were administered by continuous i.d. infusion, since continuous administration of enteral nutrition during intestinal failure is thought to enhance enteral absorption by maximizing saturation of the (residual) carrier proteins, thereby increasing intestinal function [8]. Furthermore, we previously showed that continuous enteral glucose administration improved glucose absorption during mucositis in the rat, compared with an oral bolus of glucose [23].

The median systemic availability of 4 out of 5 enterally administered AA was similar in MTX-treated rats and in controls, as found before for leucine [10], suggesting normal absorption of continuously administered AA during mucositis. However, individual availability varied from severely reduced to not different from controls among individual MTX-treated rats, despite the fact that most rats (7 of 9) suffered from severe mucositis. We hypothesize that these large interindividual differences in AA absorption during mucositis can be explained by the fact that individual MTX-treated outbred rats might have been in different stages of mucositis [as described by Sonis et al. [1]] during the AA absorption experiment. Absorption of continuously administered AA could have been possible via AA transporters on the recovered epithelial membrane, via residual transporters on damaged epithelial membrane [9] and/or via paracellular absorption [44]. Leakage of AA through damaged tight junctions could also have been possible since mucositis often leads to increased gut permeability [45, 46]. Individual AA availability often did not correlate with villus length or plasma citrulline concentrations. Although plasma citrulline was earlier shown to be a useful surrogate marker for mucositis and for malabsorption of lactose during mucositis [instead of intestinal histology [9]], we showed that plasma citrulline is not a useful marker for AA absorption during mucositis.

Apart from the systemic availability of enterally administered AA, we measured their utilization for absolute protein synthesis in small intestinal mucosa, plasma albumin, liver and thigh muscle to learn whether a potentially reduced plasma AA availability would be the result of AA malabsorption from the intestinal lumen or of increased AA

utilization (by first-pass splanchnic utilization or by utilization after becoming systemically available). A third explanation for reduced plasma AA availability would be increased AA oxidation. Although we did not measure AA oxidation, others found whole body leucine oxidation to be similar between patients with and without mucositis [10]. Because the utilization of enterally administered leucine, lysine, and phenylalanine for tissue protein and albumin synthesis was mostly lower or similar in MTX-treated rats, compared with controls, reduced systemic AA availability in some MTX-treated rats (≤21% of intake for all AA in 4 of 9 rats, Supplemental Figure 1) was probably caused by AA malabsorption. The enrichment of threonine and/or methionine was too low to measure.

In contrast to absolute synthesis, fractional synthesis of intestinal proteins and plasma albumin with enterally administered leucine, lysine and phenylalanine was higher in MTX-treated rats than in controls. A relatively increased AA utilization for intestinal protein synthesis during mucositis might indicate an increased renewal of the intestinal mucosa, which seems plausible after initial chemotherapy-induced intestinal damage [47]. However, intestinal inflammation during mucositis might also cause an increased synthesis of inflammatory proteins, such as myeloperoxidase (MPO) [9]. Albumin synthesis might be increased during mucositis to compensate for the reduced albumin concentrations that we measured. Reduced albumin concentrations during mucositis were probably caused by increased losses via the intestine as seen with other gastroenteropathies like colitis ulcerosa [48]. In general, the rat seems in a catabolic state during mucositis as can be concluded from weight loss and increased protein breakdown (containing leucine and methionine), as found previously [10]. Furthermore, peripheral protein synthesis in thigh muscle per kilogram body weight might have been reduced in MTX-treated rats, compared with controls, but could not be calculated because the total amount of muscle per rat was unknown.

We hypothesized that there could be a preferential side of the intestine for AA uptake during mucositis. In MTX-treated rats, protein synthesis in proximal and distal small intestinal mucosa with enterally administered leucine, lysine, phenylalanine and threonine was higher when taken up basolaterally than when taken up apically, indicating preferred AA uptake from the systemic side for intestinal protein synthesis during mucositis. Others have shown that enterocytes near the crypt-villus junction prefer systemically available AA, whereas enterocytes at villus tips prefer AA at the luminal side for protein synthesis [22]. Therefore, AA absorption by the intestine during mucositis was probably mainly performed by enterocytes at the crypt-villus junction, which is compatible with the observation that villi of MTX-treated rats were atrophied and damaged while crypts were already regenerating [as shown in this study and seen before by us and others [9, 47, 49]]. Also in controls, AA uptake for intestinal protein synthesis was preferred with AA originating from the systemic side, as found by others [12, 17], although differences seemed less pronounced than in

MTX-treated rats, especially in the proximal mucosa. Normally, intestinal AA absorption is very efficient [50]: the proximal small intestine (i.e. duodenum and proximal jejenum) absorbs almost all intraluminal AA, thereby leaving few AA to be absorbed in the distal small intestine (i.e. distal jejunum and ileum). The distal small intestine is therefore mainly dependent on systemically available AA, except for some AA at the luminal side that become available by recycling (proteolysis and reabsorption) of intestinal proteins [12].

If we extrapolate our findings to the clinic, they imply that enteral AA administration by continuous infusion could be useful for a substantial portion of mucositis patients, in order to improve their nutritional state, recuperation, and survival [4-6, 51]. Furthermore, enteral nutrition could possibly accelerate intestinal recovery since intraluminal nutrients have a stimulatory effect on intestinal epithelial cells and the production of trophic hormones [52-54]. Our results show that AA are important for mucositis patients so that they can meet their need for AA for intestinal protein synthesis and albumin synthesis.

We determined AA absorption during mucositis at d 4 after injection with MTX or saline. However, symptoms of mucositis are actually present during a longer period of time; from d 2 until 5 after injection with MTX [9]. We do not know whether the observed AA malabsorption in a portion of mucositis rats is structural (present on all days during mucositis) or temporal (only present on d 4). When AA malabsorption is structural, indeed only a portion of mucositis patients would benefit from continuous enteral AA administration. However, if AA malabsorption is temporal, all mucositis patients might benefit from continuous enteral AA administration during mucositis. Future studies should focus on studying the effects of continuous enteral AA administration in mucositis patients. If only a portion of patients benefit from continuous enteral AA, a marker that distinguishes between mucositis patients with a good or poor AA absorptive capacity would be highly desirable to anticipate which patients would benefit the most from continuous enteral AA administration. For now, parenteral AA administration might be a rational alternative for enteral AA administration to guarantee optimal AA availability in all patients with mucositis.

In conclusion, we show that continuous enteral administration can enable normal AA absorption in rats with chemotherapy-induced gastrointestinal mucositis. The intestine prefers basolateral AA uptake to meet its need for AA for protein synthesis during mucositis. So, although the gut might be usable for AA uptake in at least a portion of mucositis patients, when enterally administered continuously, parenteral AA administration might be a rational alternative to guarantee optimal AA availability in all patients with mucositis.

SUPPLEMENTAL METHODS

The capacity to absorb enterally administered amino acids (AA) during mucositis was studied by determining their systemic availability and utilization for (fractional and absolute) protein synthesis. To determine the systemic availability of enterally administered AA, we first determined the rate of turnover, or flux, of enterally (intraduodenally, i.d.-) and intravenously (i.v.-) infused tracers. Then, the first-pass splanchnic utilization and resulting systemic availability of enterally administered AA was determined. After the systemic availability was known, the amount of protein breakdown [indicating catabolism] could also be determined.

Isotopic enrichment of plasma AA (in mole percent excess) was used to calculate the flux (Q, in $mmol \cdot kg^{-1} \cdot h^{-1}$). The AA flux obtained with i.d.-administered tracers (^{13}C-labeled, Table 1) and i.v.-administered tracers (^{2}H- or ^{15}N-labeled, Table 1), the determination of first-pass splanchnic utilization (in % of AA intake) and resulting systemic availability of i.d.-administered AA (i.e. AA intake - first-pass uptake, in %) were calculated as previously described [10, 13]. Protein breakdown (in $mmol \cdot kg^{-1} \cdot h^{-1}$) was calculated by using the following equation:

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$Q_{i.v.}$ = Intake + Breakdown [10, 13]

where $Q_{i.v.}$ is the flux of the i.v.-administered tracer ($mmol \cdot kg^{-1} \cdot h^{-1}$) and Intake represents the systemic availability (in % of intake) of i.d.-administered AA (in $mmol \cdot kg^{-1} \cdot h^{-1}$). So, breakdown was calculated by:

Breakdown = $Q_{i.v}$ - (systemic availability x infusion rate of i.d.-administered AA)

The utilization of enterally administered AA for protein synthesis was expressed by the fractional synthesis rate (FSR) and by the absolute synthesis rate (ASR) of protein. Both the FSR and ASR were determined in proximal and distal small intestinal mucosa, plasma albumin, liver and thigh muscle. The FSR (in %/d) reflects the percentage of the total protein pool that is newly synthesized per day, and therefore indicates the relative need for AA for protein synthesis. Values were calculated as previously described [30, 55]. The isotopic enrichment of AA in tissues and albumin at baseline was assumed to be 0 mole percent excess. Normally, the enrichment of the intracellular free (unbound) AA pool (for tissues, i.e. small intestinal mucosa, liver, thigh muscle) or the plasma AA enrichment (for plasma albumin) are used as precursors. However, since the enrichment of intracellular free AA turned out to be extremely low in our tissues [possibly due to proteolysis as a result of freezing and thawing], we used the plasma AA enrichment as a precursor for small intestinal mucosa, liver and thigh muscle. The ASR (in g/d) reflects the total amount of protein that is newly synthesized per day, and therefore indicates the absolute need for AA for protein synthesis. It was measured as the FSR multiplied by the protein mass of

the organ in g/L (plasma albumin), in g/kg organ (thigh muscle, since only a sample of this muscle was collected) or in g/kg body weight (intestinal mucosa and liver).

We also aimed to determine the preferential side of the intestine to take up enterally administered AA for protein synthesis during mucositis. The i.v. infusion of AA leads to exclusive intestinal uptake from the systemic side (basolaterally). However, during i.d. infusion of AA, the intestinal uptake of enterally administered AA is from the luminal side (apically) but, after transport by the enterocyte into the systemic pool, also from the systemic side (basolaterally). Thus, by the end of the dual-tracer infusion, there are two populations of labeled AA in the small intestinal mucosa: i.d.-administered ^{13}C-labeled AA derived from both the luminal and the systemic side, and i.v.-administered ^{2}H- or ^{15}N-labeled AA derived directly from the systemic side (Table 1). The enrichment of ^{13}C-labeled AA absorbed from the systemic side (E[^{13}C]AA-$_{basolateral}$) was calculated by using the fraction of plasma [^{2}H or ^{15}N]AA incorporated into mucosa as precursor pool, as described before [56]:

$$E[^{13}C]AA_{basolateral} = E[^{13}C]AA_{plasma} \times (E[^{2}H \text{ or } ^{15}N]AA_{mucosa} / E[^{2}H \text{ or } ^{15}N]AA_{plasma})$$

where $E[^{13}C]AA_{plasma}$ is the enrichment of [^{13}C]AA in plasma, $[^{2}H \text{ or } ^{15}N]AA_{mucosa}$ is the enrichment of [^{2}H or ^{15}N]AA in intestinal mucosa and $E[^{2}H \text{ or } ^{15}N]AA_{plasma}$ is the enrichment of [^{2}H or ^{15}N]AA in plasma.

Then, the enrichment of [^{13}C]AA in mucosa absorbed from the luminal side ($E[^{13}C]AA_{apical}$) was calculated by:

$$E[^{13}C]AA_{apical} = E[^{13}C]AA_{mucosa} - E[^{13}C]AA_{basolateral}$$

where $E[^{13}C]AA_{mucosa}$ is the total [^{13}C]AA enrichment in intestinal mucosa, i.e. from both the apical and the basolateral side.

To calculate the intestinal FSR with i.d.-administered AA taken up from the systemic side ($FSR_{basolateral}$), $E[^{13}C]AA_{plasma}$ was used as a precursor. To calculate the intestinal FSR with i.d.-administered AA taken up from the luminal side (FSR_{apical}), the enrichment of [^{13}C]AA in the i.d. infusate was used a precursor.

As mentioned before, the enrichment of intracellular free AA was too low to use as a precursor. Instead, we used AA enrichment of plasma or of the intraduodenal infusate as precursors, which were probably higher than enrichment of intracellular free AA would have been, because of intracellular dilution after AA uptake from the plasma or intestinal lumen. Therefore, calculated FSR's and ASR's might be somewhat underestimated. However, differences between MTX-treated rats and controls are expected to be similar, regardless of the precursor that is used.

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