Decreased plasma and tissue isoleucine levels after simulated gastrointestinal bleeding by blood gavages in chronic portacaval shunted rats

S W M Olde Damink, C H C Dejong, N E P Deutz, P B Soeters

Abstract

**Background**—Previously, arterial concentrations of the essential branched chain amino acid isoleucine (Ile) were found to have decreased by more than 50% after gastrointestinal haemorrhage in patients and after intragastric blood administration in healthy humans and pigs. Hypothetically, this induced hypoisoleucinaemia could deplete tissue Ile pools.

**Aims**—To study the effect of repeated blood gavages on arterial and tissue Ile levels during normal and impaired liver function.

**Subjects**—Male Wistar rats.

**Methods**—14 days after portacaval shunting or sham surgery, rats received 3 ml bovine erythrocytes or saline at 0, 1, 2, and 3 hours via a gastrosotomy cathether in the duodenum. At 0, 2, 4, 6 and 8 hours arterial blood and at 8 hours intestine, liver, muscle, and cerebral cortex were sampled for determination of ammonia and amino acid concentrations.

**Results**—In both groups repeated blood administration resulted in a marked decrease in plasma Ile (40–60%). This was accompanied by decreased tissue Ile concentrations in liver (50%), muscle (40–60%), and cerebral cortex (40–50%), but unaltered intestinal Ile levels. In contrast, the arterial and tissue concentrations of ammonia, urea, and of most amino acids increased, most strikingly of the other two branched chain amino acids, valine and leucine.

**Conclusions**—Simulated gastrointestinal bleeding by blood gavages in rats with and without impaired liver function leads to hypoisoleucinaemia and decreased tissue Ile pools.

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Keywords: rats, ammonia, isoleucine, branched chain amino acids, liver failure, gastrointestinal haemorrhage.

Upper gastrointestinal bleeding is known to induce profound malaise, especially in patients with liver failure. In these patients, gastrointestinal bleeding results in high plasma ammonia levels and often precipitates hepatic encephalopathy. This association between elevated arterial levels of the toxic metabolite ammonia and the occurrence of encephalopathy has led to the hypothesis that ammonia plays a crucial role in the pathogenesis of hepatic encephalopathy. First evidence for this “toxic”, probably ammonia related, potential of a protein load in the gut during liver failure was the “meat intoxication” syndrome observed in Eck fistula dogs by Hahn and colleagues in 1893. Since then a hierarchy in the ammoniagenic potential of different proteins has been described. More specifically in patients with liver disease, enteral administration of whole blood was more ammoniagenic than a casein hydrolysate meal and a meal of packed erythrocytes led to higher ammonia levels than an isonitrogenous amount of plasma.

Observations by our group after upper gastrointestinal bleeding in patients with normal and impaired liver function, and after blood meals to healthy volunteers and pigs, led to the hypothesis that the ammoniagenic potential of (simulated) gastrointestinal bleeding is related to the complete absence of the essential branched chain amino acid (BCAAs) isoleucine (Ile) in the haemoglobin molecule, constituting about 95% of erythrocyte protein. In all these studies a more than 50% decrease in plasma Ile was observed after (simulated) gastrointestinal bleeding, while the concentrations of the other two BCAAs valine (Val) and leucine (Leu) reached extremely high levels.

Simultaneously, the arterial concentrations of most other amino acids and of ammonia and urea increased. After an isonitrogenous control meal Ile exhibited a normal postprandial increase just as all other amino acids, while the elevation of arterial ammonia and urea levels was much less pronounced compared with the blood meal, suggesting a relation between the low arterial Ile concentration and the rise in ammonia and urea levels. This relation was confirmed by the finding that when Ile was supplemented intravenously after intragastric blood administration in pigs, the hypoisoleucinaemia was prevented and the rise in arterial ammonia and urea was blunt.

These observations led to the hypothesis that the induced hypoisoleucinaemia after (simulated) gastrointestinal haemorrhage might affect the tissue free amino acid pools of Ile. This could impair tissue protein synthesis, thereby leading to a net catabolic state. Since gastrointestinal bleeding is life threatening in patients with liver insufficiency, we chose here to study rats with normal and impaired liver function. Portacaval shunting in rats was used as a model of impaired liver function, since it is well known to lead to liver disuse atrophy.
and resulting liver disfunction. In the present study arterial, intestinal, liver, muscle, and cerebral cortex concentrations of ammonia and amino acids were measured after repeated enteral administration of blood protein in rats that had received a chronic portacaval shunt, or a sham operation.

Methods

ANIMALS
Male specified pathogen free Wistar rats (n=24; 330 (SD 30) g, random bred Wistar/CPB:WU/Bor, Winkelmann, Borchen, Germany) were housed under standard conditions (not reallocated for at least 1 week, 12 hour light-dark cycle) and received standard rat chow (SRMA 20, Hopefarms, Woerden, The Netherlands) and water ad libitum until surgery. All animal use procedures were performed by licensed personnel and in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, as applied in our institute.11

EXPERIMENTAL GROUPS
Before surgery, rats were randomly assigned, based on a tail number and computer generated random tables, to one of two experimental groups.

Portacaval shunt group
This group consisted of 12 rats in whom portacaval shunting (PCS) was performed applying Funovics’ button technique using polyethylene buttons (ID 0.17 cm, OD 0.23 cm) as described recently.12 After resuscitation from surgery, these PCS rats were placed in metabolic cages with free access to standard rat chow and water. Their daily food intake was recorded and this amount was administered to their individual sham mates (pairfeeding) to eliminate the effects of the differences in food intake normally seen between PCS and sham operated rats.13

Sham group
These 12 rats underwent laparotomy and manipulations as in PCS rats, but no shunting was performed (sham operation). Rats were allowed to recover, placed in metabolic cages, and paired with their individual PCS mate. All rats were studied 14 days after surgery (see below).

SURGICAL PROCEDURES
All operations were performed in overnight fasted rats at room temperature under ether anaesthesia at constant body temperature. Portacaval shunting and sham surgery were performed as previously described.12

Fourteen days after sham or portacaval shunt surgery, the right femoral artery was catheterised for blood sampling with a heparinised PE-50 catheter (Intramedic polyethylene tubing PE-50, ID 0.058 cm, OD 0.096 cm, Clay Adams, Parsippany, NJ, USA) and fixed in place with silk ligatures. For enteral administration of bovine packed erythrocytes or saline, a gastrostomy catheter (ID 0.10 cm; OD 0.18 cm; Tygon Westvaco, Cleveland, OH, USA) was placed with its tip in the duodenum via a purse string suture in the stomach. Hereafter, all catheters were sealed and tunnelled subcutaneously to the neck, where they were exteriorised. The rats were placed back in their cages and allowed to recover. Finally, rats of each group were, again based on their tail number, randomly assigned to subgroups receiving either blood or saline meals.

BLOOD/SALINE ADMINISTRATION AND SAMPLING (SEE FIG 1)
Bovine erythrocyte packed cells were prepared by a single centrifugation step and stored at −70°C before use (one batch, protein amount 180 g/l). Bovine erythrocytes were used to simulate the bleeding, because, as in human erythrocytes, both the α14 and the β15 chain of bovine haemoglobin are completely deficient in isoleucine, whereas rat α16 and β17 chains

![Diagram](http://gut.bmj.com/)

Figure 1: Chronological sequence of the experiments.
contain some isoleucine molecules. Two hours after catheter insertion, at 0, 1, 2, and 3 hours, the conscious and unrestrained rats received either 3 ml of bovine erythrocytes or 3 ml of saline at room temperature through the gastric catheter.

At 0, 2, 4, 6, and 8 hours, before intragastric blood or saline administration, 0.5 ml arterial blood was slowly sampled (at a rate of 1:0 ml/min) in heparinised cups (Microvette LH CB1000, Sarstedt, Nümbrecht, Germany). At 0 and 8 hours, for blood pH measurements, 0.4 ml of arterial blood was sampled anaerobically and determined immediately on a blood gas analyser (ABL520, Radiometer, Copenhagen, Denmark). Sample volumes were substituted with normal saline at 37°C via the arterial catheter. Immediately after completion of the sampling, at 8 hours, tissue samples were harvested. The small bowel was grasped at Treitz’s ligament, freed from its mesentery and flushed with normal saline after which approximately 10 cm of jejunum was freeze clamped with Wollenberg tons cooled in liquid nitrogen. Hereafter, the left liver lobe, the left gastrocnemius muscle, and finally the cerebral cortex of one hemisphere were rapidly excised, freeze clamped in liquid nitrogen, and stored at −70°C.

### BIOCHEMICAL ANALYSIS

After sampling, blood was promptly kept on ice. Within 10 minutes, centrifugation was performed at 4°C at 8800 g for 5 minutes. A volume of 100 μl of plasma for amino acids was deproteinised with 4 mg sulphosalicylic acid, immediately put into liquid nitrogen, and stored at −70°C. Ammonia and urea were determined spectrophotometrically in plasma by standard enzymatic methods on a centri-fugal analyser system (Cobas Bio, Roche Diagnostica, Hoffmann-La Roche, Basel, Switzerland) using commercial kits as detailed previously. Plasma amino acids were determined with a fully automated HPLC system after pre-column derivatisation with ortho-phthalaldehyde. Water content was determined and calculated as described previously.

### STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS/PC+ Statistical Software Package, version 3 (SPSS Inc, Chicago, IL, USA). Comparisons were made between sham and PCS rats within the blood or saline subgroup to test for group effects. Similarly, comparisons were made within the sham and PCS group between blood and saline administration to test for treatment effects. For the arterial data, analysis of variance (two way ANOVA) and for the tissue data, the Mann-Whitney U non-parametric test was used. Time effects within groups were tested by one way analysis of variance. Data are presented as means (SEM); p≤0.05 was considered significant (see Tables I and II and Figs 2–4). Results on changes in behaviour and on brain neurotransmitter amino acids in this model of simulated gastrointestinal bleeding by blood gavages are discussed elsewhere.

### RESULTS

During 14 days of pairfeeding PCS and sham rats had similar food intake (not shown) and body weight (day 0: 335 (8) g v 332 (4) g; day 14: 257 (9) g v 270 (7) g). At postmortem examination all portacaval shunts proved to be patent. Overall mortality was 16.7% (n=4), all due to postoperative complications on day 1 (n=2) or day 14 (n=2).

### Isoleucine (Tables I and II and Figs 2–4)

Two hours after repeated blood gavages the arterial Ile levels were significantly decreased in both sham and PCS rats to approximately 50% of initial values (Fig 2). In the control groups arterial Ile increased after saline supplementation. Repeated enteral blood administration did not change the intestinal tissue Ile concentration (Fig 3). However, in the other organs repeated blood gavages led to a decrease in tissue Ile levels in both the sham and the PCS groups (Figs 3 and 4). This apparent decrease in tissue Ile concentration did not reach statistical significance for sham rats in liver tissue.

### Valine and Leucine (Tables I and II and Figs 2–4)

Blood administrations caused a marked increase in arterial Leu and Val concentrations in both sham and PCS rats, whereas saline supplementation caused no change. In both groups the sum of the tissue concentrations of Val and Leu increased in the liver and the brain and in PCS rats also in the intestine (Figs 3 and
TABLE II  Tissue concentrations expressed as means (SEM) in μmol × kg wet weight⁻¹ (tew)  

<table>
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<th>Sham (n=6)</th>
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<td>26248 (2292)</td>
<td>20780 (978)</td>
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<td>3092 (840)</td>
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<td>926 (81)</td>
<td>937 (58)</td>
<td>1209 (96)*</td>
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<tr>
<td>Ile</td>
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<td>25 (8)*</td>
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<tr>
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<td>99 (10)</td>
<td>132 (19)*</td>
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<td>AAA</td>
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<tr>
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<td>27577 (1012)</td>
<td>29012 (464)</td>
<td>28582 (473)</td>
<td>32543 (1409)*</td>
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AAA = aromatic amino acids Tyr+Phe. aAN = the sum of the individual amino acids measured. Symbols: Mann-Whitney U test for differences between groups: blood v saline: *p<0.05, **p<0.01, ***p<0.001; within saline or blood group: sham v PCS: *p<0.05, **p<0.01, ***p<0.001.

4). In these organs, the concentrations of Val and Leu separately showed significant increases or tendencies to increase (Table II). In muscle, however, no treatment effect on tissue Leu concentration, and as a consequence the sum of Val and Leu, could be shown.

OTHER OBSERVATIONS

Ammonia

As expected, intragastric blood meals resulted in higher arterial ammonia levels than saline meals in both groups (Table I). However, only in PCS rats did this result in a significant increase with time of the already raised arterial ammonia levels. With the exception of the liver, tissue ammonia concentrations were increased after repeated blood gavages in PCS rats, whereas sham rats showed no treatment effect (Table II).

Urea

In sham rats, blood meals led to elevated arterial urea concentrations compared with saline controls (Table I). No difference could be observed in PCS rats.

Glutamine

Only in PCS rats repeated blood gavages led to an increase in arterial glutamine levels compared with their saline mates (Table I). With the
Figure 3: Intestinal (left panels) and liver (right panels) tissue concentrations of Ile (upper panels) and the sum of Val and Leu (lower panels) in sham and PCS rats following intragastric saline (open bars) or blood administration (filled bars). Data are means (SEM) (n=4–6 per subgroup). Symbols: Mann-Whitney U test differences between blood and saline subgroups *p<0.05, and †p<0.05 for differences between sham and PCS mates.

Figure 4: Muscle (left panels) and cerebral cortex (right panels) tissue concentrations of Ile (upper panels) and the sum of Val and Leu (lower panels) in sham and PCS rats following intragastric saline (open bars) or blood administration (filled bars). Data are means (SEM) (n=4–6 per subgroup). Symbols: Mann-Whitney U for differences between blood and saline subgroups *p<0.05.

exception of the liver, this hyperglutaminemia in the PCS blood group was associated with higher tissue glutamine levels compared with the PCS saline and sham controls (Table II).

Sum of the amino acids (αAN)
In both groups, plasma and tissue levels of most amino acids increased or remained unchanged after repeated blood gavages (Tables
Decreased plasma and tissue isoleucine levels after simulated gastrointestinal bleeding by blood gavages in chronic portacaval shunted rats

I and II). Repeated blood administrations led to a similar increase in arterial aAN in sham and PCS rats (Table I). In the PCS rats only, this resulted in increased brain and intestinal tissue aAN concentrations.

Acid-base status
No differences in blood pH levels were observed between the experimental groups at 0 and 8 hours (data not shown).

Discussion
The present study was undertaken to explore further the hypothesis that the occurrence of hyperammonaemia and uremia after upper gastrointestinal bleeding is causally related to the absence of Ile in blood protein. As pointed out previously, this absence of Ile in blood protein would contribute to a decrease in plasma Ile levels after gastrointestinal bleeding. This, in turn, could hypothetically affect tissue Ile levels and consequently impair protein synthesis, contributing to a net catabolic state. If this hypothesis were correct, then it could be worthwhile to study the possibilities of treating hyperammonaemia after gastrointestinal bleeding, which is a serious complication in cirrhotics, by giving Ile simultaneously during gastrointestinal bleeding.

We examined part of the hypothesis by studying arterial and tissue amino acid concentrations. Ile decreased in arterial plasma after gastrointestinal bleeding, as we have previously observed in various species during normal and impaired liver function.6-7 Also, in full accordance with our hypothesis (simulated) gastrointestinal haemorrhage by repeated intragastric blood administrations resulted in a decrease in tissue Ile concentrations in liver, muscle and brain of approximately 40-60% of control values in both PCS and sham rats. However, intestinal tissue Ile concentration remained unaffected.

Blood protein (mainly haemoglobin) is almost completely deficient in the essential amino acids Ile and methionine.7-8 Since repeated blood gavages did not change plasma or tissue methionine levels (see Tables I and II), the observed decrease in tissue and plasma Ile concentrations cannot solely be explained by the absence of Ile in the blood protein administered via the enteral route. It is probably also related to the simultaneous presence of high amounts of the other BCAAs, Val and Leu, in blood protein.9 This BCAA imbalance in the administered blood protein can lead to a phenomenon called BCAA antagonism, presumably resulting from shared BCAA transport and/or degradation routes (reviewed in Harper et al10-12).

Of the latter mechanisms involved in BCAA antagonism, competition for transmembrane transport by the markedly elevated Val and Leu with the decreased Ile concentrations does not seem to play a major role in the present study, since Ile levels were similarly decreased in blood and tissue.

Another, more likely, explanation for the Ile decrease is the fact that all BCAAs initially have a shared degradation pathway.14, 25 Within the cell, the initial step is a reversible, concentration dependent, transamination reaction by BCAA aminotransferase (CAT; EC 2.6.1.42). Hereafter, the resulting 2-oxoacids undergo an irreversible oxidative decarboxylation reaction by the branched-chain 2-oxoacid dehydrogenase complex (BCODC, EC 1.2.4.4), the rate limiting step in BCAA oxidation.26 Henceforth, the reaction products of the individual BCAAs follow their own catabolic pathway, for example, to the Krebs cycle. BCODC activity shows large differences between various organs because it is present in active (high content in liver) and in inactive forms (high content in muscle, brain, and intestine),24, 25 the latter being most potently activated by the 2-oxoacid of Leu.

Accordingly, Leu concentrations in blood protein could stimulate BCAA oxidation by activating the rate limiting step of the common degradation pathway, irrespective of the level of the other BCAAs.22 Consequently, Ile oxidation will be stimulated, resulting in a further depression in plasma and tissue Ile levels, as shown in the present study. The observed decrease in tissue Ile concentrations will probably be even more pronounced intracellularly, since tissue consists of approximately 15% extracellular water26, 27 and plasma Ile levels exceeded tissue levels at the end of the study. Tissue Ile depression may be even more severe in the first 2 hours after intragastric blood gavages, when plasma levels were most decreased.

In the present study we did not observe a decrease in Ile concentration in intestinal tissue. This can be explained either by a low BCAA oxidation rate as both CAT and BCODC activity are very low in the intestine,28 or by net catabolism of intestinal protein. Earlier work from our group, reporting enhanced release of amino acids, especially Val and Leu, in the portal vein after a blood meal compared with an isonitrogenous control meal provides support for a role of protein breakdown in the unchanged Ile concentration in the intestine.7 Also, a decrease in Ile might be prevented by enhanced splanchnic uptake of Ile, which occurs after Leu infusions.29

Interestingly, the simulated gastrointestinal bleeding did not increase arterial urea levels in PCS rats, although plasma ammonia and glutamine were markedly increased. This could point to a decreased urea synthesis capacity in these animals, as was shown by Steele2 in rats 14 days after portacaval shunting. The mechanism of this reduction in urea synthesis capacity cannot be deduced from our study, but could be explained by a reduction in liver weight and portacaval shunting or by shunting of the small periportal area of hepatic glutaminase activity.30

The hypothesis that low tissue Ile concentrations could diminish protein synthesis is consistent with other reports concerning impaired protein synthesis, DNA synthesis, cell proliferation, and cell function in an Ile deficient state.31-35 Impaired protein synthesis could shift the balance between protein
synthesis and breakdown towards a net catabolic state. Lecailler et al. showed that even a short period (8 hours) of hypoinsulinemia impaired whole body protein synthesis in humans. Hypothetically, the synthetic activity of proteins was diminished for a short half-life, as for instance cloting factor VII (4 hours), could be affected in such a short hypoinsulinemia period.

Of therapeutic interest is our previous observation that supplementing Ile intravenously after simulated gastrointestinal bleeding in normal pigs normalised the plasma Ile levels and blunted the rise in arterial ammonia and urea levels. We hypothesised that Ile infusion replenished the lacking Ile and thereby improved protein synthesis in various organs, resulting in a net anabolic state and less amonniagenesis. Infusion of Ile after gastrointestinal bleeding could, therefore, hypothetically reduce the incidence of hepatic encephalopathy in patients with impaired liver function and cirrhosis in pregnant women. In future studies we will focus on supplementing Ile as a therapeutic intervention during upper gastrointestinal haemorrhage in humans with and without impaired liver function.

In conclusion, the present study shows that simulated gastrointestinal bleeding by blood gavages during normal and impaired liver function in rats leads to hypoinsulinemia and a decrease in tissue Ile pools in brain, muscle, and liver to approximately 40–60% of initial values. However, it had no influence on intestinal tissue Ile concentration. Depletion of Ile pools in the body could impair protein synthesis and therefore the function of rapidly dividing cells and short half life proteins, especially those with high Ile content.

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