Amino Acids (2008) 35: 225–231
DOI 10.1007/s00726-007-0557-5
Printed in The Netherlands

Amino Acids

Serum amino acid profile in patients with acute pancreatitis

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Received November 14, 2006
Accepted April 20, 2007
Published online May 23, 2007;

Summary. Patients in the early phase of acute pancreatitis (AP) have reduced serum levels of arginine and citrulline. This may be of patho-biological importance, since arginine is the substrate for nitric oxide, which in turn is involved in normal pancreatic physiology and in the inflammatory process. Serum amino acid spectrum was measured daily for five days and after recovery six weeks later in 19 patients admitted to the hospital for acute pancreatitis. These patients had abnormal levels of most amino acids including arginine, citrulline, glutamine and glutamate. Phenylalanine and glutamate were increased, while arginine, citrulline, ornithine and glutamine were decreased compared to levels after recovery. NO_2/NO_3 concentration in the urine, but not serum arginase activity, was significantly increased day 1 compared to day 5 after admission. Acute pancreatitis causes a disturbance of the serum amino acid spectrum, with possible implications for the inflammatory process and organ function both in the pancreas and the gut. Supplementation of selected amino acids could possibly be of value in this severe condition.

Keywords: Pancreas – Acute pancreatitis – Nitric oxide – L-arginine – L-citrulline

Introduction

Disturbed amino acid balance may have negative effects in pancreatitis and other severe conditions (Luiking et al., 2004). Patients with acute pancreatitis have reduced levels of serum arginine and citrulline in the early phase of the disease (Sandström et al., 2003). Arginine is the substrate for production of nitric oxide (NO) *via* the enzyme nitric oxide synthase (NOS), which exists in three subforms. NOS uses tetrahydrobiopterin (BH_4) as a cofactor (Boucher et al., 1999; Alderton et al., 2001), and the same cofactor is also used in the conversion of phenylalanine to thyrosine (Alderton et al., 2001). Neurogenic NOS (nNOS) is important for sphincter relaxation (Thune et al., 1995), and selective inhibition of nNOS increases contractions in the sphincter of Oddi (Sandstrom et al., 2004). Endothelial NOS (eNOS) is found mainly in caveolae in the endothelial cells of the vascular wall, and is important for relaxation of blood vessels (Shaul, 2002). The third isoform, inducible NOS (iNOS), is activated by TNFα, IL6 and LPS (Hallemeesch et al., 2002; Vallance, 2003; Wu and Morris, 1998).

In experimental pancreatitis, iNOS activity is greatly increased (Ayub et al., 2001), and some studies indicate that NO production correlates with the severity of acute pancreatitis in humans (Mettu et al., 2003; Rahman et al., 2003). This could be due to a direct effect of a high iNOS induced production of NO and reactive nitrogen species (RNS). A sudden and strong consumption of arginine by iNOS could possibly also reduce the substrate available for eNOS and nNOS, which in turn would aggravate organ dysfunction. Production of NO by iNOS is an intracellular process but is regulated by the extracellular levels of arginine, as the metabolism is closely linked to the cell membrane transporter, cationic amino acid transporter, CAT (Hallemeesch et al., 2002). As other cationic amino acids like ornithine and lysine use the same transporter, these may act as competitive inhibitors of arginine (Hallemeesch et al., 2002).

Arginine metabolism includes a number of enzymes and pathways (Fig. 1). In one of these pathways, catalysed by the enzyme arginase, arginine is converted to ornithine and urea. Arginase exists in two forms, type 1 found mainly in the liver and involved in the urea cycle, and type 2 found in a number of tissues like kidney, brain, small intestines and macrophages (Boucher et al., 1999). Arginase competes with NOS for arginine as a substrate (Bansal and Ochoa, 2003) and the low arginine levels in

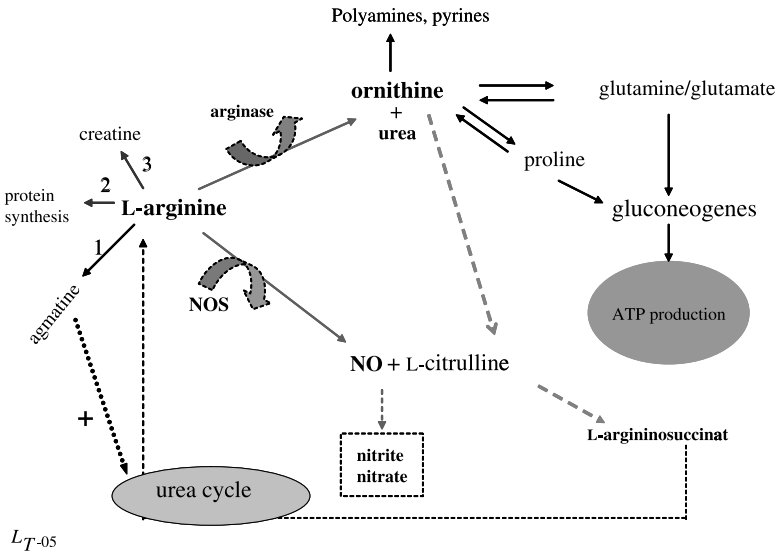

Fig. 1. The metabolism of L-arginine. Ninety per cent of the arginine comes from dietary intake and protein turnover, and the rest comes from *de novo* synthesis *via* citrulline that is formed in the gut and converted to arginine in the kidneys. Arginine is metabolised in 5 different pathways: NOS, Arginase, Arginine decarboxylase (1), Arginyl t-RNA synthetase (2) and arginine:glycine amidinotransferase (3)

the early phase of acute pancreatitis could be due to an increased arginase activity. Ornithine is further metabolised to glutamine, which is important for gut integrity (De-Souza and Greene, 2005). Infection in necrotic pancreatic tissue is the main cause of death in human acute pancreatitis, and the bacteria originate from the gut, implying that gut integrity is of vital importance.

As in septic conditions, the body is in a catabolic state in acute pancreatitis, with a general negative effect on amino acid balance (Luiking et al., 2004). The pool of amino acids in the circulation is small compared to the whole body, and the serum levels may change over short periods of time. In the present study we tested the hypothesis that during the first five days of acute pancreatitis, amino acid metabolism could influence the inflammatory process and organ function in general. We also evaluated serum arginase activity and the production of nitrite/nitrate to determine the importance of these two catabolic pathways for arginine during acute pancreatitis. The results were compared to the situation in the same patients after recovery six weeks later.

Materials and methods

Patients

The study was approved by the Ethics Committee for investigations involving human subjects at Linköping University and included 19 patients (4 females, 15 males), median age 66.5 (32–84) years, with *acute pancreatitis* who were admitted to Linkoping University Hospital and Vrinnevi Hospital, Norrkoping, during 2005. Acute pancreatitis was defined as serum pancreatic amylase activity more than three times the upper limit of the reference range, accompanied by abdominal pain. Patient characteristics and the results of laboratory tests are shown in Tables 1 and 2. The patients were also grouped according to severity of their clinical condition (Table 2). Severe acute pancreatitis was defined as necrotizing pancreatitis and/or organ failure lasting longer than 48 h, a modification of the Atlanta classification (Bradly, 1993). Nine patients, 7 females and 2 males, had gallbladder stones verified by ultrasonography, and no history of alcohol abuse, and were diagnosed as *gallstone pancreatitis*.

Table 1. Patient characteristics

	All ($n=19$)	Gallstone ($n=9$)	Alcohol ($n=7$)	Hyperpara ($n=1$)	Idiopathic ($n=2$)
Age	66.5(32–84)	66.5(48–81)	57(32–81)	84	64.5(62–67)
Men	15	7	7	0	1
Women	4	2	0	1	1
CRP max (mg/l)	194(25)	190(33)	245(43)	124	72(8)
WBC max (10^9/l)	15.6(1.3)	16.3(1.8)	16.0(2.6)	11.2	15.6(1.8)
Amylase (ukat/l)	14.8(2.8)	15(4.3)	16.8(4.5)	10.8	5.9(2.8)
Lipase (ukat/l)	22.2(4.8)	26.8(6.6)	13.7(1.9)	5.7	14.6(4.7)
US (gallstone)	9	9	0	0	0
Creatinine (mmol/l)	100(5)	92(5)	111(11)	116	85(14)
APACHE II max	11(5–22)	10(5–16)	12(6–22)	11	9(9)

Nineteen patients with acute pancreatitis. Patient characteristics separated according to aetiology
Normal serum values for C-reactive protein (CRP <10), white blood cells (WBC, 4–9), amylase (0.2–0.8), lipase (0.36–0.85), creatinine (55–100)
US, Ultrasound examination

Table 2. Patient characteristics

	Mild	Severe
Age	62.5(45–84)	67(32–69)
Men	11	4
Women	4	0
CRP max (mg/l)	162(90)	315(38)**
WBC max (10^9/l)	13.8(1.0)	22.4(2.4)**
Amylase (ukat/l)	14.2(3.6)	16.6(2.5)
Lipase (ukat/l)	22.3(5.7)	21.8(2.5)
Creatinine (mmol/l)	94(4)	121(15)*
Urea (mmol/l)	6(0.8)	6.5(1.8)
APACHE II (max)	9(5–14)	15(12–22)**

19 Patients with acute pancreatitis. Patient characteristics according to severity
* $p < 0.05$, ** $p < 0.01$. Normal serum values for C-reactive protein (CRP <10), white blood cells (WBC, 4–9), amylase (0.2–0.8), lipase (0.36–0.85), creatinine (55–100) and urea (3.0–9.0)

They had a maximal mean (SEM) CRP of 195(25) mg/l, a white blood cell count (WBC) of 15.6(1.3) 10^9/l, and an APACHE II median score of 10 (range 5–16). Seven male patients had a history of alcohol misuse and were diagnosed as *alcoholic pancreatitis*. They had a maximal CRP of 245(43) mg/l, a WBC count of 16.0(2.6) 10^9/l, and an APACHE II score of 12 (6–22). One female patient had acute pancreatitis due to primary hyperparathyroid disease with a CRP of 124, a WBC count of 11.2 and an APACHE II score of 11, and one male and one female had *idiopathic pancreatitis,* with a CRP of 72(8), a WBC count of 15.6(1.8), and an APACHE II score of 9(9). The patients were on their regular diet before start of the acute phase, and had had symptoms for 3–48 h before hospital admission. Patients with severe pancreatitis had higher maximum levels of CRP, WBC, creatinine and APACHE II scores than patients with mild pancreatitis. During the five-day study period the patients were fasted and received infusions of saline and glucose 5%; no proteins or lipids were given. The patients were back on their regular diet at hospital discharge.

Blood and urine sampling

Urine and blood samples were taken daily, upon admission and for the five-day study period. The blood was immediately centrifuged for 10 min at 3000 rpm and stored at −20 °C until analysed. At recovery six weeks after hospital discharge, urine and blood samples were taken after an overnight fast.

Amylase and lipase in serum

Serum α-amylase activity was measured according to Kruse-Jarres et al. (1989) and serum lipase was assessed according to Panteghini and Bonora (1996).

Nitrite and nitrate in urine

The sum of nitrite and nitrate in urine was assessed using a colorimetric method based on the Griess reaction for nitrite, following enzymatic conversion of nitrate with nitrate reductase from Aspergillus (Gilliam et al., 1993). For the assay, 0.1 ml of urine was diluted with 5 ml of phosphate buffered saline (PBS), pH 7.3. After addition of 0.05 ml nitrate reductase (1 mU/l, Roche Diagnostics) and nicotinamide adenine dinucleotide phosphate, 0.05 ml, and reduced NADPH (1.8 mmol/l), the reaction mixture was incubated at room temperature for 30 min. This mixture was used for the Griess assay of nitrite by adding 0.25 ml of sulphanilamide (0.1 M in hydrochloric acid) and naphthylethylenediamine 0.25 ml (8 mmol/l). The resulting colour was read in a spectrophotometer (Beckman DU 68) at 540 nm (Gilliam et al., 1993).

Arginase activity in serum

Arginine hydrolysis was carried out in Eppendorf tubes in a final volume of 200 µl, containing 0.5 M L-arginine pH 9.7, 1 mM $MnCl_2$, and 100 µl of serum. Incubation was performed at 37 °C for 30 min, and the reaction was stopped by the addition of 800 µl of an acid mixture. After addition of 100 µl of 3% isonitrosopropiophenone (dissolved in absolute ethanol), samples were heated at 100 °C for 30 min and maintained in the dark for 10 min, before the absorbance of the urea formed was measured at OD, 540 nm, using a spectrophotometer. The calibration curve was prepared with increasing amounts of urea between 1.5 and 120 µg. One unit (U) of enzymatic activity was defined as the amount of enzyme that catalyses the formation of 1 µmol urea/min. Arginase activity in serum was expressed in units per litre of serum (del Ara Rangel et al., 2002).

Amino acid analysis

Twenty-three amino acids were quantified in morning serum samples for the first five consecutive days and at the follow-up after six weeks. Free amino acids were analysed by an anion-exchange chromatographic method combined with spectrophotometric quantification (amino acid analyser Biotronik LC 3000, Centre for Inherited Metabolic Diseases, Karolinska University Hospital, Huddinge, Stockholm), (Hagenfeldt and Arvidsson, 1980). The serum level of each amino acid is given as the mean ± SEM and the results are compared within each patient. The normal ranges used in the paper are from the same lab (Hagenfeldt and Arvidsson, 1980).

Statistical analysis

Results are expressed as means ± SEM if not otherwise stated. Parametric data were tested by Student's two-tailed paired *t*-test and one-way Analysis of Variance (ANOVA) + Tukey-Kramer Multiple Comparisons. Non parametric data were analysed with the Mann Whitney *U*-test and correlation analyses were done with Person's correlation coefficient, with a p value less than 0.05 considered statistically significant.

Results

Clinical characteristics

History of recurrent disease and clinical course are shown in Table 3. Two patients with acute biliary pancreatitis, one with acute alcoholic pancreatitis and one with idiopathic pancreatitis, had had previous episodes of pancreatitis. No major differences were seen between the groups regarding length of hospital stay. Two of the patients with alcohol pancreatitis were treated in the intensive care unit for 3 and 11 days, respectively. Four of the patients were classified as having a severe attack of acute pancreatitis (Bradly, 1993), three with alcohol and one with gallstone pancreatitis.

Urinary nitrite and nitrate

Urinary concentrations of NO_2/NO_3 were measured daily for five days after the onset of symptoms and at the fol-

Table 3. Patient characteristics

	All ($n=19$)	Gallstone ($n=9$)	Alcohol ($n=7$)	Hyperpara ($n=1$)	Idiopathic ($n=2$)
Hospital stay in days. median (range)	7.5(4–27)	9(4–27)	8.5(4–19)	7	4.5(4–5)
No of deaths	0	0	0	0	0
No treated in ICU	2	0	2	0	0
Previous episode of acute pancreatitis	4	2	1	0	0
Previous episode of cholecystitis	0	0	0	0	0
Severe acute pancreatitis	4	1	3	0	0

Patient characteristics according to aetiology and severity. Severe pancreatitis was defined as CT verified necrotizing pancreatitis or organ failure >48 h

Table 4. Selected serum amino acids, arginase and CRP in patients with acute pancreatitis

	Normal range	Day 0	Day 5	Recovery
CRP (mg/l)	<10	41(12)	148(26)***	14(4)
Arginase (U/l)	0.30–0.85	1.22(0.20)	0.94(0.08)	0.96(0.10)
Ornithine (μM)	20–105	59(7)**	61(4)***	83(5)
Arginine (μM)	30–125	88(7)**	101(8)*	115(4)
Citrulline (μM)	15–50	22(2)***	19(2)***	39(3)
Glutamine (μM)	355–725	355(18)***	331(36)***	531(18)
Glutamate (μM)	10–60	198(30)**	181(36)**	106(11)

All 19 patients with acute pancreatitis. Comparison of CRP and arginase activity between days 1 and 5; for arginase activity the difference is not significant, ($p=0.12$). Amino acids days 1 and 5 compared with recovery, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. The Mann Whitney test was used for analysis of amino acids and the *t*-test for CRP and arginase activity

low-up after six weeks. An increased urinary NO_2/NO_3 concentration was seen at admission as compared to each of the following days in hospital (Fig. 3).

Amino acids and arginase activity in serum

The serum levels of CRP, arginase activity and the amino acids arginine, citrulline, ornithine, glutamine and glutamate are shown in Table 4. Upon admission (day 0), patients with acute pancreatitis had reduced serum levels of arginine, mean 88.5(SEM 7) compared to the levels after recovery, 115(4), $p<0.01$; and of citrulline, 22(2), compared to recovery 39(3), $p<0.001$; and of ornithine, 59(7), compared to recovery 83(5), $p<0.01$; and of glutamine, 355(18), compared to recovery 531(18), $p<0.001$. These differences remained during the five days of the study. The level of glutamate was increased during the first five days of acute pancreatitis, day (0), 198(30), compared to recovery 106(11), $p<0.01$. The arginase activity in serum, normal range 0.30–0.85, was increased at day (0), mean 1.22(SEM 0.20), compared to day 5, 0.94(0.08), but did not reach statistical significance ($p=0.14$), (Table 4). The serum levels of the other amino acids analysed are shown in Table 5. Only taurine, aspartate, α-aminobutyrate and tryptophan were at the same level at day (0) as at recovery. Phenylalanine was increased compared to the recovery level, $p<0.001$, while all other amino acids were reduced. The branched chained amino acids, valine, leucine and isoleucine, were all reduced at day (0) but normalised at day (5) compared to

Table 5. Amino acids in patients with acute pancreatitis

Amino acids	Normal range (μM)	Day 0	Day 5	Recovery
Taurine	30–80	92(10)	75(7)	92(4)
Aspartate	0–15	25(4)	27(4)	21(1)
Threonine	90–195	91(6)***	112(7)	124(7)
Serine	75–170	103(7)*	117(7)	120(5)
Asparagine	20–100	36(3)**	37(3)*	48(2)
Proline	95–270	128(9)***	139(10)***	272(21)
Glycine	140–340	195(11)**	207(17)	243(13)
Alanine	185–455	389(29)*	292(26)***	464(17)
a-Aminobutyrate	10–40	18(1)	26(2)***	19(1)
Valine	175–300	219(8)***	246(11)	268(9)
Methionine	15–35	24(1)***	29(1)	31(3)
Isoleucine	40–85	52(4)***	75(5)	84(3)
Leucine	100–160	138(7)*	148(9)	161(7)
Tyrosine	30–75	56(3)***	54(3)***	78(5)
Phenylalanine	40–70	93(5)***	82(4)*	69(2)
Lysine	120–220	142(7)***	164(12)*	192(6)
Histidine	65–115	72(4)**	58(3)***	87(4)
Tryptophan	30–65	52(3)	47(3)*	57(3)

Serum amino acids in the 19 patients with acute pancreatitis, at admission (day 0), (day 5) and at recovery after 6 weeks. The values are means (SEM) and the unit are μM

$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ are significance levels for comparisons with the recovery level using paired *t*-tests

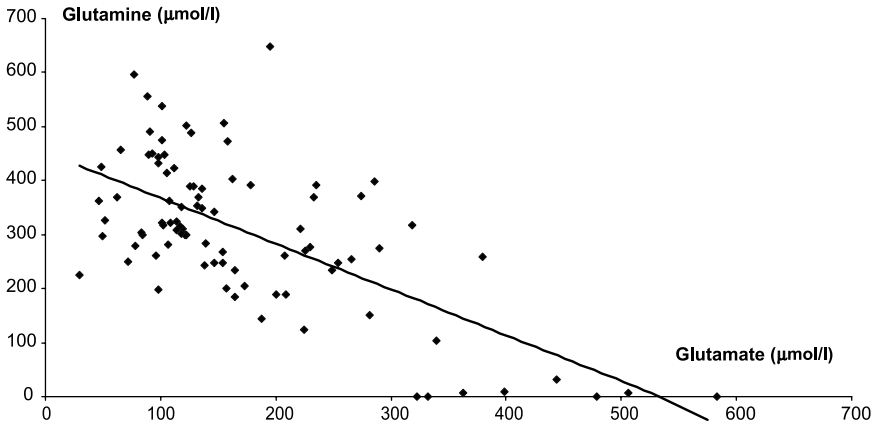

Fig. 2. Correlations of serum levels of glutamine and glutamate in 19 patients with acute pancreatitis. The serum levels had a negative correlation with $r = -0.663$ and $p < 0.01$

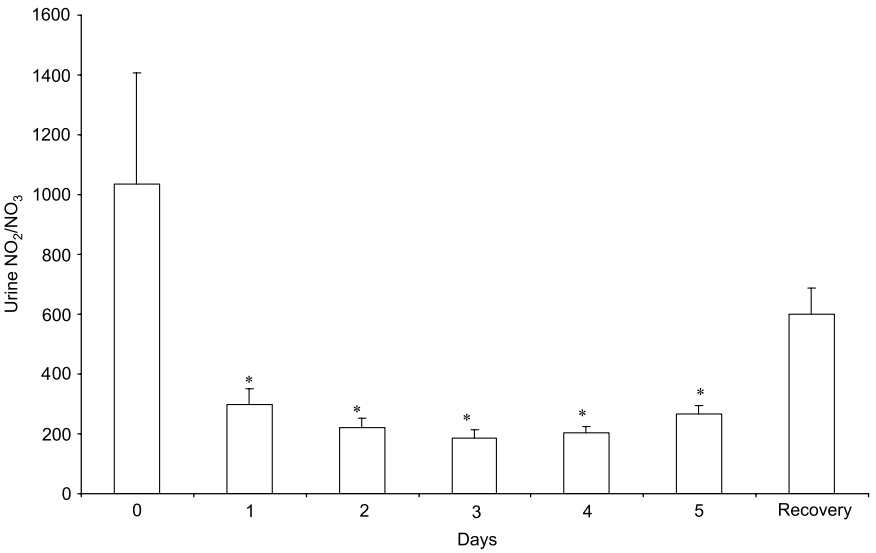

Fig. 3. Urinary levels of nitrite/nitrate in 19 patients with acute pancreatitis on admission, during 5 days of hospitalisation and at recovery (6 weeks post-hospitalisation). The levels were increased at day 0 ($^*p < 0.05$) compared to day 1–5, but were not significantly different from recovery levels ($p = 0.14$)

recovery. No differences in amino acid patterns were found between patients with mild and those with severe pancreatitis.

Analysis of correlations between amino acids and arginase activity

The serum levels of glutamine and glutamate were negatively correlated, $r = -0.66$ ($p < 0.01$) (Fig. 2). There were no significant correlations between serum levels of amino acids and arginase activity.

Discussion

This observational study shows that patients admitted to a hospital with acute pancreatitis, irrespective of cause, have low serum concentrations of L-arginine, L-citrulline, ornithine, and glutamine, and increased levels of glutamate and phenylalanine compared to the situation six weeks later.

The serum amino acids originate from food intake or by release from tissues following proteolysis or synthesis. The amino acids disappear from serum by tissue uptake and, to a negligible extent, through body losses. The uptake of amino acids into the cells is mainly an active process mediated by transporters that are specific for different groups of amino acids. A number of factors including hormones and food intake regulate, normally strictly, the serum amino acid levels and the plasma pools of amino acids adjust quickly.

In the early phase of acute pancreatitis, serum concentrations of most amino acids were low, including the branched chain amino acids that are released from skeletal muscles where they are usually metabolised. During the five-day study the patients were only given infusions of glucose 5% and saline. Infusion of glucose may increase serum insulin levels, leading to a decrease of most plasma amino acids. This is most pronounced for the branched amino acids, since insulin blocks the release of these from muscle. These mechanisms may partly explain the differ-

ences in amino acid concentrations during the first five days compared to those after recovery.

The patients had a negative nitrogen balance during the monitored five days in hospital, as they were not fed and were only given infusions of saline and glucose (Rand et al., 2003). The downregulation of ureagenesis is due at least in part to the low level of serum arginine (an activator of the urea cycle) with reduced flux to the liver. Instead, arginine is metabolised to citrulline in the small bowel and then converted back to arginine in the kidney. In this way arginine is saved from being converted to urea and lost (Cynober et al., 1995).

During the course of the first five days after hospital admission the serum arginine and citrulline levels remained low and were not normalised, which could have several explanations. Arginine transport is mainly mediated by transporter (y+, CAT). This transporter is activated by several factors such as endotoxin, LPS, IL-1 and TNF-α (Luiking et al., 2004), and it has been shown that synthesis of the transporter is quickly induced in vascular endothelium and gut epithelium by these factors. This facilitates an increased production of NO *via* iNOS induction in acute pancreatitis (Mettu, 2003; Rahman, 2003). At the same time, this may reduce NO formation *via* eNOS and nNOS (Luiking et al., 2004), an effect which may last during the five-day follow-up. Reduced formation of NO by eNOS and nNOS may be detrimental in acute pancreatitis as it may reduce pancreatic vascular perfusion and inhibit relaxation of the sphincter of Oddi.

An increased NO production has been shown in both human and experimental pancreatitis (Ayub et al., 2001; Mettu et al., 2003; Rahman et al., 2003) and may enhance intestinal permeability and serum endotoxin levels (Rahman et al., 2003). As the patients did not eat, serum arginine would be derived from protein degradation and reformation from citrulline. Since serum citrulline levels remained low, reformation of arginine might have been impaired in these patients.

Serum arginase activity tended to be increased, although not significantly, at admission compared to day five and to six weeks later (Table 4). It is well known that NOS and arginase compete for arginine as a substrate. Normally one of the two enzymes is activated, but some stimuli like LPS may activate both (Bansal and Ochoa, 2003), and LPS is reported to be increased in acute pancreatitis (Rahman et al., 2003). Arginase is mainly associated with the wound healing process, and an increased activity would be expected at the end of the study period. The correlation between the serum arginine level and arginase activity, however, was weak, indicating that arginase may not be the main factor regulating the arginine serum level in this situation. Recently it has been shown that cytokine stimulation may differentiate monocytes into either M1 or M2 macrophages (Mantovani et al., 2002). Arginine metabolism is influenced by both the high levels of iNOS forming NO and citrulline in M1 macrophages, and the arginase pathway which predominates in the M2 macrophages with generation of ornithine and polyamines. An increased macrophage activity in the acutely inflamed pancreatic tissue could consume serum arginine at a rate that may not be compensated.

NOS needs tetrahydrobiopterin (BH_4) as a co-factor when NO is produced (Alderton et al., 2001; Boucher et al., 1999). BH_4 is also necessary for the conversion of phenylalanine to thyrosine by the enzyme phenylalanine hydroxylase (Alderton et al., 2001). High NO production may consume or bind most of the BH_4, leading to a reduced conversion of phenylalanine to thyrosine, and may explain the increased serum levels of phenylalanine and reduced levels of thyrosine. The quotient between the serum levels of phenylalanine and tyrosine was shifted during acute pancreatitis compared to after recovery. Normally this quotient is below one (0.9 at recovery), but at admission, i.e. during the attack, and at day 5, it significantly increased to values of 1.7 and 1.5, respectively ($p < 0.001$).

The metabolisms of glutamine and glutamate are closely interconnected and involved in a number of metabolic pathways like formation of other amino acids, GSH, ammonia and urea (Nissim, 1999). Glutamine is also important for gut integrity, which in turn is of great importance in acute pancreatitis. Bacteria from the gut may infect the inflamed and necrotic pancreatic tissue, thereby constituting one of the main causes of death in acute pancreatitis. The normal glutamine:glutamate ratio is 5–10:1, but in acute pancreatitis patients it was 1.8:1. Intracellular acidosis could be one possible underlying factor explaining this shift in the glutamine/glutamate ratio (Nissim, 1999).

This observational study suggests that the patho-biology of acute pancreatitis may interfere with the serum amino acid profile. Several mechanisms may contribute to explaining this biochemical disturbance that may influence the inflammatory events and organ functions.

Acknowledgements

A special thanks to Marie Stenlund for the collection of samples and data, and to Tony Forslund for the analysis of arginase activity.

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