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Effects of diazepam on neutrophil (PMN) free amino acid profiles and immune functions in vitro. Metabolical and immunological consequences of L-alanyl-L-glutamine supplementation

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Abstract

The objective of this study was to determine the effects of diazepam, L-alanyl-L-glutamine (ala-gln) or diazepam combined with ala-gln on polymorphonuclear leukocyte (PMN) free amino acid profiles. In a parallel study the effects on PMN immune functions were also documented for the first time. The incubation of whole blood with diazepam led to significant changes in PMN free glutamine, aspartate, glutamate, ornithine, arginine, citrulline, taurine and methionine as well as branched chain and neutral amino acid concentrations. Ala-gln caused significant increases in PMN glutamine and alanine and asparagine, aspartate, glutamate, ornithine, arginine, serine and glycine profiles. Regarding PMN immune functions, diazepam significantly decreased superoxide anion (O_2^-) and hydrogen peroxide production (H_2O_2) and myeloperoxidase activity (MPO) while ala-gln significantly increased PMN immune functions. Ala-gln supplemented to diazepam largely reversed the changes in PMN amino acid profiles and PMN immune functions brought about by diazepam. Overall, diazepam or ala-gln lead to significant changes in PMN free amino acids. Important PMN immune functions also seem to be affected. In regards to the results, there is significant relevance to the pharmacological regimens which enhance the supply of diazepam or ala-gln in whole blood suggesting that considerable changes in PMN "labile free amino acid pool" occur. These regimens often follow beneficial nutritional therapy or maleficent pharmacological stress and may be one of the determinants in cell nutrition which influence PMN function. It is partially through its effect on PMN labile free amino acid pool that ala-gln supplemented to diazepam may maintain PMN immune functions in vitro.

Keywords: Diazepam; L-alanyl-L-glutamine; Neutrophils; Amino acids; Immune function

1. Introduction

Initial resistance to bacterial infection is mediated primarily by polymorphonuclear leukocytes (PMN). Their importance become particularly apparent when their numbers are reduced or their functions become impaired. This is because derangement of any PMN function may allow bacterial infection to develop and might contribute to patient's morbidity and mortality [1]. Several studies have shown that diazepam, which plays an important part in modern neurology and psychiatry in the treatment of epilepsia, tetanus or anxiety, can alter immunological defense by modifying the number and/or functions of immunocompetent cells, including PMN, with impairment of microbicidal activity [2,3]. These effects may do not necessarily lead to immunosuppression, but they might potentially further compromise already depressed host defense mechanisms if used in critically ill patients. Increasing evidence suggest that especially the "labile free intracellular amino acid pool" is important to metabolic and physiological state of PMN as well as to the special functions in the inflammatory response of these cells [4,5]. Until now, however, only limited knowledge is available regarding the influences of diazepam

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on PMN free amino acid profiles [6]. Important findings suggest that glutamine-dipetides especially (L-alanyl-L-glutamine) may have beneficial pharmacological value in modulating cellular metabolism as well as the immune response in rapidly proliferating cells such as PMN. L-alanyl-L-glutamine has been shown to play important roles in cell-mediated immune functions and received immense interest in promotion of antimicrobial as well as PMN antitumoral effector systems. Various studies suggest that L-alanyl-L-glutamine may be an important substrate especially for immunocompetent and rapidly proliferating cells, and may be required in increased amounts when catabolic conditions prevail [7,8,9]. However, the major biochemical mechanisms by which L-alanyl-L-glutamine modulates major cellular functions, PMN amino acid concentrations and important PMN immune functions have not been defined yet. Moreover, our current understanding of the rate of L-alanyl-L-glutamine utilization and major pathways of metabolism raises some intriguing questions concerning possible therapeutic manipulation and whether specific important PMN antibacterial host defense mechanisms can be beneficially altered or not. Consequently, an important alternative proposal is the consideration of L-alanyl-L-glutamine as a "tissue-specific" single nutrient.

Summing up: at the present state of knowledge, no information is available concerning the effects of either diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine on PMN "labile free intracellular amino acid pool" as well as on important PMN immune functions [as obtained by superoxide anion generation, hydrogen peroxide formation and myeloperoxidase activity]. If L-alanyl-L-glutamine is protective, and diazepam damaging to immune cell function, it would also be useful to determine whether L-alanyl-L-glutamine might influence any potentially effects induced by diazepam.

The goals of this study were therefore:

1. to document the effects of diazepam, L-alanyl-L-glutamine or diazepam combined L-alanyl-L-glutamine with on free PMN amino acid concentrations. With regard to clinical relevance we have chosen an experimental design with whole blood, followed by rapid and highly selective enrichment of PMN, thereby preserving high cellular viability and integrity.
2. In a parallel study the effects of a diazepam, L-alanyl-L-glutamine or diazepam combined L-alanyl-L-glutamine incubation of whole blood on released myeloperoxidase activity and both superoxide anion and hydrogen peroxide formation in isolated PMN were also investigated.

2. Materials and methods

The study was approved by the local ethics committee of the Justus-Liebig-University, Giessen. Eighteen male volunteers between 24 and 32 years (mean 27 years) with an average height of 181 cm (range 175–190) and weight of 80 kg (range 70–97) were selected: those volunteers with metabolic (diabetes etc.), cardiopulmonary, neurological or allergic diseases, or volunteers taking drugs were not accepted to the study. Whole blood samples (Lithium-heparinate plastic tubes) were withdrawn between 08:00 and 09:00 (10 hr of fasting) to exclude potential circadian variations.

2.1. Diazepam, L-alanyl-L-glutamine or diazepam+ L-alanyl-L-glutamine

The following concentrations were tested:

1. Diazepam: (Valium MM Roche®, Hoffmann La Roche, Grenzach-Wyhlen, Germany): 0; 0.4; 4 and 40 $\mu g \cdot mL^{-1}$
2. L-Alanyl-L-glutamine (Dipetamin® [200 g L-alanyl-L-glutamine $\cdot L^{-1}$; (= 82 g L-Alanyl, =134,5 g L-glutamine; = 0,92 mol $\cdot L^{-1}$ L-alanine and 0,92 mol $\cdot L^{-1}$ L-glutamine)], Fresenius, Bad Homburg, Germany): 0 and 5 Mol L-alanyl-L-glutamine
3. Diazepam + L-alanyl-L-glutamine: 0 and 0.4; 4 or 40 $\mu g \cdot mL^{-1}$ diazepam + 5 mMol L-alanyl-L-glutamine respectively

The lesser diazepam concentration is that which immediately appears in serum after intravenous injections of clinically relevant diazepam (10 mg) doses [10]. The higher diazepam concentrations corresponded to 10 and 100 times the clinically achieved plasma concentrations. The selected L-alanyl-L-glutamine concentration corresponded to 10 times physiological plasma concentrations (see [4] for physiological values). With regard to the clinical relevance originally L-glutamine was omitted from incubation because of both *1)* its intravenous application in patients is not licensed and *2)* application of L-glutamine is connected with important chemical and galenic problems such as chemical instability (decomposition of glutamine is not controllable and depends on temperature, the pH value and concentration of anions in the incubation medium for example), low water solubility and fears of its rapid breakdown to pyroglutamate and ammonium which may influence PMN function as well. L-alanyl-L-glutamine dipeptide is not connected with such chemical and galenic problems. L-alanyl-glutamine is rapidly hydrolyzed by plasma hydrolases, also during in vitro incubation, and has been found to be a suitable source for glutamine with comparable biological activity [11,12,13]. Solutions of diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine were prepared and diluted in Hank's balanced salt solution (HBSS; Sigma, Deisenhofen, Germany) and the pH in the test solution confirmed to be 7.4. 1 ml of whole blood was incubated with 25 μl of test solution (the final concentrations were as described) for two hours at 37°C (vibrating water bath). The corresponding volumes of HBSS were added to the control tubes.

2.2. Highly selective separation of polymorphonuclear leukocytes (PMN) from whole blood

Precise details of our PMN-separation technique have been described previously [14,15]. This method is a further development of the methods described by Eggleton et al. [16] which allows a very rapid and selective enrichment of PMN preserving high cellular viability and integrity from very small quantities of whole blood. Extraction of PMN was accomplished using a cooled (4°C) Percoll®-gradient. The cooled and heparinized whole blood samples were then overlaid into a previously prepared and precooled (4°C) Percoll®-gradient before centrifugation. After this cellular separation the PMN were carefully taken from the sample and suspended in cooled (4°C) and diluted PBS® stock buffer. After a second centrifugation, the PBS® stock buffer was discarded and the erythrocytes remaining in the sample were hypotonically lysed using cooled (4°C), destilled water. After 20 s the PMN samples were immediately brought back to isotonicity and resuspended by adding diluted PBS® stock buffer. After a third centrifugation the PBS® stock buffer was again discarded and 2 aliquots of resuspended sample were removed. Immediately after withdrawal and preparation, the extracted and cooled PMN samples were deep frozen at −80°C before lyophilization (freeze drying at −80°C). In aliquots of the PMN samples both the purity as well as their viability were subsequently verified by light microscopy. Samples with a PMN purity $< 96\%$ and those with more than 4% avitality were discarded.

2.3. Chromatographic amino acid analysis

Amino acids in PMN were quantified using previously described methods which fulfill the strict criteria required for ultrasensitive, comprehensive amino acid analysis which was especially developed and precisely validated in our institute for this purpose (see [15] for details). The lyophilizates were solubilized in 80% methanol/20% H_2O so as to guarantee short term stability before the column derivitization procedure ("chemical preservation"). This standard extraction buffer also contained the amino acid l-homoserine as an internal standard for the HPLC procedure. Standard samples (including all amino acids which we measured in PMN) have been prepared in our laboratory and methanolic extracts of the various lyophilized samples were prepared as previously described [15]. After a 5 min incubation and 3 min centrifugation (3000 × g, Rotixa/KS®, Tuttlingen, Germany), samples were transferred to a special sample tube (2-CRV®, Chromacoll, Trumbull, U.S.A) where alkaline 0.5 M borate buffer (Merck, Darmstadt, Germany) and *o*-phthaldialdehyde-2-mercaptoethanol (OPA; Merck, Darmstadt, Germany) were automatically added. This base derivatization was stopped after exactly 120 s by neutralization with 0.75 N HCL (Merck, Darmstadt, Germany). The mixture was then transferred to a rarefaction vial and diluted with eluent A, and 25 μl of this mixture was injected into the columns. The program and the automatically degassed (3-channel degasser, Knauer, Berlin) solvents used were as previously described [15]. The flow rate was maintained at 1.0 ml/min throughout by using a hydrostatic gradient pump. The fluorescence high performance liquid chromatography system (F-HPLC) consisted of a hydrostatic gradient pump, a controller for gradient programming (600 E®, Waters, U.S.A.) and a programmable autosampler for the automated derivatization procedures (Triathlon®, Spark, The Netherlands) within a rheodyne injection valve and a 100 μl filling loop (AS 300®, Sunchrom, Germany). The following column was used for separation: Nova-Pak®, 300 mm × 3,9 mm I.D.; RP-C-18, 60 Å, 4 μm (Waters, U.S.A.). Column-temperatures were maintained at 35°C using a column oven (Knauer, Germany). Fluorescence was routinely monitored using a fluorescence spectromonitor (RF-530®, Shimadzu, Japan). Measurements were made at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. Data recording and evaluation was performed using computer integration software (EuroChrom 2000® Knauer, Germany). The coefficients of variations for both method reproducibility as well as reproducibilities of the retention times were as described elsewhere [15].

Superoxide anion and hydrogen peroxide production, and activity of released myelo-peroxidase (MPO), were photometrically determined using significant modifications of known methods precisely validated in our institute for this purpose [17,18]. Superoxide anion production was measured by reduction of cytochrome C. 100 mg of cytochrome C (type IV, Sigma, Deisenhofen, Germany) which was dissolved in 30 ml PBS®-glucose buffer. The solution was portioned and frozen at −20°C. Opsonized zymosan (Sigma, Deisenhofen, Germany) was used to stimulate PMN. It was produced by incubating 100 mg zymosan with 6 ml pool serum for 30 min at 37°C. After washing with saline and centrifuging at 350 × g (10 min) opsonized zymosan was re-suspended in 10 ml PBS®-glucose buffer, portioned and frozen at −20°C. Whole blood was incubated either with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested for 2 hours at 37°C. The PMN were then isolated using a modification of our PMN-separation technique (as mentioned above). After stepwise (15 min and 5 min) centrifugation procedures (350 × g, 20°C) as well as careful lysis of a few erythrocytes contaminating the pellet, the PMN-cells were resuspended by adding diluted PBS® (Gibco, Karlsruhe, Germany) stock buffer. After 7 ml PBS® stock buffer had been administered, the tube was centrifuged at 350 × g for 5 min (20°C). The supernatant was decanted. Samples with a PMN purity $<96\%$ and those with more than 4% dead cells were discarded. The PMN concentration required in each case was adjusted by adding PBS containing 9.99 g glucose (Merck, Darmstadt, Germany). After PMN isolation, 500 μl zymosan, 150 μl pool serum, 250 μl cytochrom C and 500 μl isolated PMN suspension ($0{,}8 \times 10^6$/ml) and again diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested, were poured into a test tube. A

preparation containing 500 μl buffer instead of zymosan was used as a zero adjustment. After further incubation for 15 min at 37°C the reaction was stopped by putting the test tube into ice water. After centrifugation (350 × g; 5 min, 4°C) extinction of the supernatant was measured photometrically (546 nm; Digitalphotometer 6114S®; Eppendorf, Germany). The amount of superoxide anion produced resulted from the extinction coefficient of cytochrome C according to the law of Lambert & Beer [19]. All control probes obtained for standard curves have been prepared, incubated and measured identically. Hydrogen peroxide production was also determined photometrically. The method based on horse radish peroxidase catalyzed by oxidation of phenol red by hydrogen peroxide. Phenol red (Sigma, Deisenhofen, Germany) and horse radish peroxidase (type II, Sigma, Deisenhofen, Germany) were added to PMN which had been stimulated by opsonized zymosan. Phenol red was dissolved in double-destilled water (10 g/L). Horse radish peroxidase was dissolved in PBS®-glucose buffer (5 g/L). After incubation of whole blood with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested for 2 hours at 37°C PMN were isolated as described above. Isolated PMN were stimulated by opsonized zymosan (Sigma, Deisenhofen, Germany). The final test preparation consisted of 500 μl zymosan, 125 μl pool serum, 12.5 μl horse radish peroxidase, 12.5 μl phenol red, 12.5 μl sodium azide (200 mmol/L/L; Merck, Darmstadt, Germany), 500 μl PMN suspension (2×10^6 PMN-cells/ml) and again diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine. After incubation for 15 min (37°C), the test preparation was centrifuged for 5 min (350 × g; 4°C). Subsequent to adding 25 μl sodium hydroxide solution (1.0 normal, Merck, Darmstadt, Germany), the extinction was measured photometrically at 623 nm. All control probes obtained for standard curves have been prepared, incubated and measured identically. Activitiy of released myeloperoxidase: 1 mmol/L 2.2′-azino-di-(3-ethylbenzthiazoline) sulfonic acid (ABTS, Sigma, Deisenhofen, Germany) was dissolved in 0.1 Mol/L citrate buffer (Behring, Marburg, Germany; pH 7,4). After incubation of whole blood with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested for 2 hours at 37°C, 100 μl isolated PMN suspension (2×10^6/ml) was incubated with 0.5 μg cytochalasin B (Sigma, Deisenhofen, Germany) and again with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine (5 min; 37°C). After adding 100 μl opsonized zymosan and supplementing in order to keep the concentration constant, the preparation was incubated again for 10 min (37°C). Then 1 ml ATBS solution was added. After centrifuging (700 × g, 5 min, 20°C) 1 ml of supernatant was removed and mixed with 1 μl hydroxide peroxide solution (30%; Merck, Darmstadt, Germany) and extinction was measured photometrically (405 nm).

2.3.1. Statistical analysis

The statistical analysis and interpretations of our study results were performed in close relationship with our Department of Medical Statistics.

2.3.1.1. PMN amino acid concentrations. All tests were performed in duplicate. Thus, our PMN amino acid results are the means of two estimations. After the results were demonstrated to be normally distributed (Pearson Stephens test), statistical methods were performed including Bartlett test to check homogeneity of variance ($p \leq 0.1$). If the requirements were met, a two-factor analysis of variance for repeated measures (ANOVA) was conducted. Probability levels of $p \leq 0.05$ versus control were considered significant. The data are given as means ± standard deviations (mean ± SD).

2.3.1.2. PMN immune functions. All tests were performed in duplicate. Thus, our results are the means of two estimations. After the results were demonstrated to be normally distributed (Pearson Stephens test), statistical methods included Bartlett test to check homogeneity of variance ($p \leq 0.1$). If the requirements were met, an two-factor analysis of variance for repeated measures (ANOVA) was conducted. If the requirements were not fulfilled, the Friedmann test was performed. Probability levels of $p \leq 0.05$ versus control were considered significant. The data are given as mean ± SD.

3. Results

The free intracellular amino acid concentrations, superoxide anion formation (O_2^-), hydrogen peroxide generation (H_2O_2) as well as activity of released myeloperoxidase (MPO) obtained in the control cells were within normal physiological ranges (see [14,15] as well as [17,18] for physiological values); (Tables 1 and 2).

3.1. Diazepam

3.1.1. Effects on amino acids

Incubation with diazepam ($\geq 0.4\ \mu g \cdot mL^{-1}$) caused significant decreases in PMN glutamine, aspartate, arginine, ornithine, citrulline, alanine, taurine and α-aminobutyrate. Moreover, following diazepam significant increases in PMN serine, glycine, isoleucine, leucine, valine, threonine and methionine concentrations have been found (Table 1). None of the other amino acids have been affected significantly.

3.1.2. Effects on superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO) activity

In the presence of diazepam ($\geq 4\ \mu g \cdot mL^{-1}$) O_2^- production, H_2O_2 formation and MPO activity significantly decreased.

3.2. L-alanyl-L-glutamine

3.2.1. Effects on amino acids

Incubation with 5mmol/L L-alanyl-L-glutamine caused significant increases in PMN free intracellular glutamine, asparagine, glutamate, aspartate ornithine, arginine, citrulline,

Table 1
Effects of diazepam (0.4 μg, 4 μg, 40 $\mu g \cdot ml^{-1}$) and 5 mMol L-alanyl-L-glutamine (Ala-Gln) on PMN labile free amino acid pool (pMol per PMN-cell; Mean ± SD) and PMN superoxide anion generation [nmol · (10^6 PML · min)$^{-1}$], hydrogen peroxide production [nmol · (10^6 PML · min)$^{-1}$] and myeloperoxidase activity [units · L supernatant^{-1}].

Abb.	Control	Diazepam			Ala-Gln 5 mMol
		0,4 $\mu g \cdot ml^{-1}$	4 $\mu g \cdot ml^{-1}$	40 $\mu g \cdot ml^{-1}$	
Acid amino acids, acid amides					
asn	0.39 ± 0.06	0.38 ± 0.06	0.40 ± 0.07	0.42 ± 0.08	0.48 ± 0.08*
gln	2.63 ± 0.59	2.77 ± 0.59	1.97 ± 0.44*	1.63 ± 0.36*	3.97 ± 0.88*
asp	2.50 ± 0.40	2.03 ± 0.36*	1.91 ± 0.32*	1.64 ± 0.26*	3.53 ± 0.58*
glu	6.01 ± 0.82	5.07 ± 0.76*	4.28 ± 0.60*	3.58 ± 0.56*	8.93 ± 1.28*
Basic amino acids					
orn	0.41 ± 0.08	0.32 ± 0.07*	0.28 ± 0.06*	0.24 ± 0.05*	0.53 ± 0.11*
lys	0.58 ± 0.10	0.69 ± 0.13	0.64 ± 0.12	0.60 ± 0.11	0.69 ± 0.14
arg	0.29 ± 0.05	0.28 ± 0.05	0.23 ± 0.05*	0.18 ± 0.03*	0.38 ± 0.06*
cit	0.11 ± 0.02	0.10 ± 0.02	0.08 ± 0.02*	0.06 ± 0.01*	0.14 ± 0.03*
Branched chain amino acids					
ile	0.31 ± 0.06	0.36 ± 0.07	0.40 ± 0.08*	0.43 ± 0.09*	0.28 ± 0.05
leu	0.60 ± 0.13	0.70 ± 0.15	0.76 ± 0.16	0.83 ± 0.18*	0.69 ± 0.15
val	0.36 ± 0.06	0.41 ± 0.07	0.46 ± 0.07*	0.50 ± 0.08*	0.38 ± 0.06
Neutral amino acids					
ser	1.38 ± 0.20	1.70 ± 0.25*	1.80 ± 0.27*	2.11 ± 0.31*	1.84 ± 0.29*
gly	2.43 ± 0.35	3.01 ± 0.48*	3.29 ± 0.59*	3.62 ± 0.64*	3.25 ± 0.46*
thr	0.60 ± 0.09	0.70 ± 0.11	0.79 ± 0.12*	0.88 ± 0.13*	0.58 ± 0.08
ala	1.71 ± 0.31	1.59 ± 0.26	1.32 ± 0.25*	1.04 ± 0.20*	2.51 ± 0.45*
Aromatic amino acids					
tyr	0.29 ± 0.06	0.29 ± 0.06	0.27 ± 0.05	0.28 ± 0.06	0.30 ± 0.06
trp	0.11 ± 0.02	0.12 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.11 ± 0.02
phe	0.77 ± 0.11	0.72 ± 0.11	0.70 ± 0.11	0.68 ± 0.10	0.73 ± 0.10
his	0.61 ± 0.20	0.69 ± 0.21	0.55 ± 0.18	0.59 ± 0.19	0.61 ± 0.19
Taurine, methionine, α-aminobutyrate					
tau	40.5 ± 7.3	36.8 ± 6.7	32.6 ± 5.9*	29.2 ± 5.4*	39.9 ± 7.1
met	0.15 ± 0.03	0.17 ± 0.04	0.19 ± 0.04*	0.23 ± 0.05*	0.15 ± 0.03
aba	0.21 ± 0.04	0.19 ± 0.04	0.17 ± 0.03	0.14 ± 0.03*	0.22 ± 0.04
Superoxide anion, hydrogen peroxide, myeloperoxidase					
O_2^-	3.259 ± 0.558	2.716 ± 0.535	2.307 ± 0.458*	1.821 ± 0.404*	5.931 ± 1.074*
H_2O_2	1.089 ± 0.227	0.902 ± 0.211	0.669 ± 0.145*	0.592 ± 0.131*	1.916 ± 0.406*
MPO	0.551 ± 0.135	0.441 ± 0.116	0.385 ± 0.103*	0.286 ± 0.079*	0.931 ± 0.261*

Abb. = Abbreviations (see text).
* = $p \leq 0.05$ versus control values.

serine, glycine and alanine concentrations (Table 1). PMN free lysine, citrulline, isoleucine, leucine, valine, methionine, threonine, a-aminobutyrate, tyrosine, tryptophane, phenylalanine, histidine and taurine profiles remained uneffected.

3.2.2. Effects on oxidative response and myeloperoxidase activity

Incubation with 5mMol L-alanyl-L-glutamine led to significant increases in superoxide anion and hydrogen peroxide production as well as myeloperoxidase activity (Table 1).

3.3. Diazepam + L-alanyl-L-glutamine

3.3.1. Effects on amino acids

Addition of 5 mMol L-alanyl-L-glutamine to 0.4 and 4 μg diazepam · mL^{-1} neutralized the effects of diazepam alone for all of the amino acids tested (Table 2). The addition of glutamine to 40 μg diazepam · mL^{-1} failed to have any marked influence on the effects of diazepam alone for most of the amino acids tested, although changes in PMN glutamine, aspartate and alanine following high-dose diazepam alone have been completely neutralized by exogenous L-alanyl-L-glutamine. Moreover, changes in glutamate and arginine have partially been counterbalanced by supplemented arginine, too (Table 2).

3.3.2. Effects on superoxide anion, hydrogen peroxide and myeloperoxidase activity

Glutamine completely reversed changes in O_2^- and H_2O_2 formation as well as MPO activity brought about by 0.4 and 4 μg diazepam · mL^{-1} alone. Glutamine supplementation to 40 μg diazepam · mL^{-1} completely counterbalanced the

Table 2
Effects of 0.4 ($D_{0.4}$); 4 (D_4) and 40 (D_{40}) μg diazepam · ml^{-1} combined with L-alanyl-L-glutamine (5 mMol; Ala-Gln) on PMN labile free amino acid pool (pMol per PMN-cell; Mean ± SD) and PMN superoxide anion generation [nmol · (10^6 PML · min)$^{-1}$], hydrogen peroxide production [nmol · (10^6 PML · min)$^{-1}$] and myeloperoxidase activity [units · L supernatant^{-1}].

Abb.	Control	$D_{0.4}$ + Ala-Gln	D_4 + Ala-Gln	D_{40} + Ala-Gln
Acid amino acids, acid amides				
asn	0.39 ± 0.06	0.41 ± 0.06	0.42 ± 0.06	0.45 ± 0.07
gln	2.63 ± 0.55	2.96 ± 0.64	2.52 ± 0.52†	2.16 ± 0.48†
asp	2.50 ± 0.40	2.45 ± 0.38†	2.35 ± 0.39†	2.12 ± 0.35†
glu	6.01 ± 0.82	5.91 ± 0.80†	5.28 ± 0.80†	4.63 ± 0.71*†
Basic amino acids				
orn	0.41 ± 0.08	0.36 ± 0.07	0.33 ± 0.06	0.27 ± 0.06*
lys	0.60 ± 0.13	0.57 ± 0.12	0.65 ± 0.12	0.62 ± 0.13
arg	0.29 ± 0.05	0.31 ± 0.07	0.27 ± 0.05	0.23 ± 0.04*,†
cit	0.11 ± 0.02	0.12 ± 0.03	0.09 ± 0.02	0.07 ± 0.01*
Branched chain amino acids				
ile	0.31 ± 0.06	0.33 ± 0.06	0.35 ± 0.06	0.40 ± 0.08*
leu	0.60 ± 0.13	0.68 ± 0.14	0.70 ± 0.15	0.78 ± 0.17*
val	0.36 ± 0.06	0.35 ± 0.06	0.39 ± 0.07	0.44 ± 0.07*
Neutral amino acids				
ser	1.38 ± 0.20	1.52 ± 0.24	1.64 ± 0.26	1.78 ± 0.25*
gly	2.43 ± 0.36	2.64 ± 0.43	2.89 ± 0.50	3.17 ± 0.46*
thr	0.60 ± 0.09	0.66 ± 0.10	0.70 ± 0.11	0.76 ± 0.12*
ala	1.71 ± 0.31	1.76 ± 0.33	1.66 ± 0.36	1.42 ± 0.28†
Aromatic amino acids				
tyr	0.29 ± 0.06	0.29 ± 0.06	0.29 ± 0.06	0.28 ± 0.06
trp	0.11 ± 0.02	0.12 ± 0.02	0.10 ± 0.02	0.13 ± 0.02
phe	0.77 ± 0.11	0.72 ± 0.11	0.78 ± 0.11	0.70 ± 0.11
his0.61 ± 0.20	0.61 ± 0.20	0.69 ± 0.22	0.70 ± 0.22	0.55 ± 0.18
Taurine, methionine, α-aminobutyrate				
tau	40.5 ± 7.3	37.9 ± 7.6	35.6 ± 6.5	32.9 ± 6.3*
met	0.15 ± 0.03	0.16 ± 0.03	0.17 ± 0.03	0.21 ± 0.04*
aba	0.21 ± 0.04	0.20 ± 0.04	0.17 ± 0.03	0.16 ± 0.03*
Superoxide anion, hydrogen peroxide, myeloperoxidase				
O_2^-	3.259 ± 0.558	3.682 ± 0.659†	2.783 ± 0.452†	2.493 ± 0.521*,†
H_2O_2	1.089 ± 0.227	1.273 ± 0.286†	0.909 ± 0.187†	0.828 ± 0.189*,†
MPO	0.551 ± 0.135	0.598 ± 0.150†	0.509 ± 0.123†	0.429 ± 0.107†

Abb. = Abbreviations (see text).
* = $p \leq 0.05$ versus control values; † = $p \leq 0.05$ versus diazepam alone.

effects of diazepam on MPO and only partially reversed its effects on O_2^- production and H_2O_2 formation (Table 2).

4. Discussion

4.1. Diazepam

Diazepam ($\geq$0,4 $\mu g \cdot mL^{-1}$) significantly decreased intracellular glutamine, aspartate, glutamate, ornithine, arginine, citrulline, alanine, taurine and α-aminobutyrate levels and increased the profiles of important other amino acids (i.e. branced chain amino acids, neutral amino acids, methionine). Only aromatic amino acids, asparagine and lysine have not been influenced. However, especially the fact that PMN free glutamine, glutamate as well as aspartate were significantly reduced, points to important mechanisms underlying the diazepam induced changes. At the present state of knowledge various findings suggest that glutamine, aspartate as well as glutamate are essential intracellular fuels for leukocytes even when resting [20]. The role of glutaminolysis, in rapidly dividing cells such as PMN in particular, has been considered to provide both the nitrogen and carbon physiologically required for precise and sensitive control for PMN intermediate (i.e. α-keto acids) and macromolecule synthesis (i.e. DNA, RNA) [21]. Changes in PMN free glutamate and aspartate concentrations may also have essential consequences for PMN respiratory fuel sources, producing energy yielding substrates, because for example both amino acids in PMN enter the citrate acid cycle [22]. Recent former findings described that glutamine, glutamate and aspartate metabolism is closely related to the specific functions in the PMN inflammatory response [23,24]. Consequently, we suggest that changes in PMN labile free intra-

cellular glutamine, glutamate as well as aspartate pool, for example induced by diazepam, may be analogous to changes in PMN glycolysis which causes maleficient intracellular conditions adversely effecting PMN function [25]. Moreover, diazepam decreased intracellular arginine, ornithine and citrulline levels. This favors the hypothesis that decreases in PMN free arginine concentrations have been followed by decreased arginine conversion into important arginine dependent amino acid metabolites [26]. This may also have essential consequences for PMN function because various findings suggest that changes in arginine turnover may underlie important cellular immune depression [27,28, 29]. Moreover, it has also been suggested that especially ornithine, which in undisturbed leukocyte metabolism is rapidly converted from arginine by argininase [30], shares the immunostimulatory and secretagogue effects of arginine [31]. Further important findings of our study show that diazepam incubation of whole blood significantly decreased PMN intracellular taurine profiles which may draw attention to changes in PMN osmoregulation [32,33,34]. Taurine accounts for approximately 55–60% of all PMN free intracellular amino acids and high intracellular concentrations are maintained against a high cell-plasma concentration gradient. Interestingly, decreases in intracellular taurine concentration has been followed by increases in PMN neutral amino acid levels which are also known to impart osmoprotection in various cells [35,36,37]. Decreases in PMN taurine profiles especially may also have important consequences for PMN function since especially taurine has been suggested to be involved in important leukocyte immune functions [38,39]. Diazepam also altered the PMN free branched chain amino acid pool (leucine, isoleucine, valine) which in leukocytes may play essential roles as regulators in PMN protein turnover [40]. Former interesting findings concerning the effects of diazepam on intracellular amino acid profiles have unfortunately been connected with essential limitations inherent in the methodology and study design, because results obtained in rats mainly reflects changes in amino acid profiles characteristically altered following prolonged starvation [6]. When sufficient nutritional supply has been changed ("..a total of 3 animals (25%) became abnormally weak.."), for example following chronic high dose diazepam treatment (8 mg · kg^{-1} body weight), the first priority of cellular metabolism is to provide glucose for the brain. Especially glucogenic amino acids, incorporated in cellular protein, can be degraded to pyruvate or to TCA-cycle intermediates to a point where the carbons could be used for the production of glucose or to rebuild glycogen. And of major importance, during starvation, only carbon skeletons acquired from amino acids really provide a net synthesis of glucose in leukocytes [4]. Regarding PMN immune functions diazepam significantly decreased both superoxide anion and hydrogen peroxide generation and myeloperoxidase activity suggesting an undisputed reduction in PMN function. Our findings confirm former investigations in which diazepam impaired neutrophil reactive oxygen species generation as assessed by free radical formation chemiluminescence or oxidative activity [41,42]. Although this study was not able to confirm any direct relationship between free amino acids and PMN immune functions or whether alterations in PMN amino acid profiles reflects direct changes of specific PMN intracellular pathways, there is significance that pharmacological regimens which enhance the supply of diazepam to whole blood considerably influences PMN labile free intracellular amino acid pool in vitro which may be one of the determinants in cell nutrition positively or adversely (i.e. following diazepam) affecting important PMN immune functions.

4.2. L-alanyl-L-glutamine

L-alanyl-L-glutamine supplementation to whole blood (5mmol/L) significantly increased free intracellular glutamine as well as alanine profiles in PMN indicating increased uptake of alanine and glutamine by PMN from plasma and/or increased uptake of L-alanyl-L-glutamine dipeptide followed by intracellular hydrolysis [43,44]. Indeed, high plasma L-alanyl-L-glutamine hydrolase activities, followed by consecutive uptake of the liberated amino acid residues, have been previously described. Moreover, various tissue and blood cells contain sufficient membrane bound as well as intracellular hydrolasis activity [45,46,47, 48,49]. Therefore, L-alanyl-L-glutamine dipeptides especially have been found to be a suitable source for glutamine and alanine with comparable biological activity [11,12,13]. Connected with these findings, we observed significant increases in further important PMN free intracellular amino acids (i.e. glutamate, aspartate, asparagine, ornithine, arginine, citrulline, serine and glycine) as well. Again, this may indicate both increased uptake of L-alanyl-L-glutamine metabolites from whole blood and/or high glutamine and alanine utilization by PMN [43,50]. In the author's opinion our findings would favor the latter hypothesis because high rates of glutamine but also alanine uptake and utilization, especially by rapidly dividing cells such as PMN, have previously been described [51,52]. Increasing evidence suggest that not only glucose but also glutamine in PMN provides and maintains both nitrogen and carbon for the synthesis of macromolecules and act as an oxidative fuel for energy production when required [20,21]. Not only glutamine but glutamate as well may have been a substratum for further metabolical processes producing neutral amino acids such as serine and glycine as well as aspartate which in PMN, just as glutamate, enters the citrate acid cycle [22]. Moreover, Metcoff et al. [53] described that a combination of PMN free aspartate, glycine, ornithine, arginine, glutamate and glutamine profiles were especially highly predictive regarding the levels of PMN energy charge. Interestingly, exogenous L-alanyl-L-glutamine supplementation also increased PMN intracellular arginine and ornithine concentrations. Regarding the pattern of PMN amino acid changes, we hypothesize that PMN, similar to macrophages or mono-

cytes, may contain sufficient activity of the enzyme systems required to convert glutamine to arginine and ornithine [54]. Moreover, L-alanyl-L-glutamine supplementation significantly increased antimicrobicidal functions of human PMN in vitro, suggesting an anabolic role of L-alanyl-L-glutamine for PMN. Until now, little information is available concerning the fuel that PMN-cells use for important immune functions, but various investigators described that essential immune functions of human leukocytes strongly depend on intracellular glutamine concentrations [55,56, 57]. Consequently, with regard to former findings we suggest that nutritional or pharmacological regimens which enhance the supply of L-alanyl-L-glutamine to PMN may have considerable pharmacological value in modulating essential PMN functions.

4.3. Diazepam + L-alanyl-L-glutamine

An important aspect of this study was to determine whether L-alanyl-L-glutamine could reverse any changes induced by diazepam. L-alanyl-L-glutamine, however, largely neutralized the changes in PMN labile free amino acid concentrations as well as essential PMN immune functions brought about by diazepam. For example, decreases in glutamine, glutamate, ornithine, arginine or citrulline and increases in branched chain amino acids have been reversed. Also of major importance was the fact that L-alanyl-L-glutamine largely reversed the changes in taurine and neutral amino acids brought about by diazepam. Only following a high dose diazepam treatment did glutamine fail to reverse the metabolic and immunologic effects sufficiently. Consequently, we belief that any large or prolonged effect on PMN labile free amino acid pool and/or any decrease in the flux through important PMN amino acid pathways ("branched point sensitivity") could affect the functioning of important PMN immune responsibilities [58]. With regards to our results we suggest that L-alanyl-L-glutamine which has maintained the concentration and composition of PMN "labile free amino acid pool" may therefore provide important "antidote" or "remedy" activity against for example maleficient pharmacological stress (i.e. diazepam) induced changes [23,24,44,53]. Summing up, our results confirm the beneficial effects of L-alanyl-L-glutamine in at least some important aspects of PMN labile free intracellular amino acid pool as well as on PMN immune function and may add further in vitro evidence for the possible advantage of including this amino acid in the advanced therapeutical standards (i.e. intensive care unit, etc.).

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