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Alterations in neutrophil (PMN) free intracellular alpha-keto acid profiles and immune functions induced by L-alanyl-L-glutamine, arginine or taurine

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Summary. The objective of this study was to determine the dose as well as duration of exposure-dependent effects of L-alanyl-L-glutamine, arginine or taurine on polymorphonuclear neutrophil (PMN) free α-keto acid profiles and, in a parallel study, on PMN immune functions. Exogenous L-alanyl-L-glutamine significantly increased PMN α-ketoglutarate, pyruvate PMN superoxide anion (O_2^-) generation, hydrogen peroxide (H_2O_2) formation and released myeloperoxidase (MPO) activity. Arginine also led to significant increases in α-ketoglutarate, pyruvate, MPO release and H_2O_2 generation. Formation of O_2^- on the other hand was decreased by arginine. Incubation with taurine resulted in lower intracellular pyruvate and α-ketobutyrate levels, decreased O_2^- and H_2O_2 formation and a concomitant significantly increased MPO activity. We therefore believe that considerable changes in PMN free-α-keto-acid profiles, induced for example by L-alanyl-L-glutamine, arginine or taurine, may be one of the determinants in cell nutrition that considerably modulates the immunological competence of PMN.

Keywords: L-Alanyl-L-glutamine – Arginine – Taurine – Neutrophil – α-Keto acids – Immune function – Superoxide anion – Hydrogen peroxide – Myeloperoxidase

Introduction

Initial resistance to bacterial infection is mediated primarily by polymorphonuclear neutrophils (PMN) as an important component of non-specific cell-mediated immunity. A basic precondition for an optimal defense against pathogenic microorganisms is an undisturbed function of all the immunocompetent PMN metabolic pathways, since a derangement in any of the PMN host defense mechanisms may allow or contribute towards patient morbidity and mortality (Liu and Pope, 2004; Witko-Sarsat et al., 2000; Gabrilovich, 1999). Increasing evidence suggest that L-alanyl-L-glutamine, arginine or taurine, may have beneficial pharmacological effects in modulating cellular metabolism as well as the immune response in rapidly proliferating cells such as PMN (Alvarez and Mobarhan, 2003; Davidson, 2004; Fürst, 1990a and b; Grimm and Kraus, 2001; Montejo et al., 2003; Singh et al., 2002).

Regarding glutamine-dipeptides, various investigators have stated that L-alanyl-L-glutamine in particular may be useful for modulating cellular metabolism as well as the immune response in rapidly proliferating cells such as PMN. This is because this dipeptide represents a suitable source for glutamine and alanine and has a comparable biological activity. (Pithon-Curi et al., 2002a and b; Newsholme et al., 2001 and 2003b). The list of biological activities associated with glutamine or alanine is impressive and includes ''nitrogen shuttle activities'' as well as key roles in cellular energetics and metabolism due to their function as important sources of cellular fuel. Moreover, both amino acids are also known to be sources of carbon for metabolic intermediates and macromolecular synthesis (Castell, 2003; Curi et al., 1999, 1997a and b; Moinard et al., 1999 and 2002a; Mühling et al., 2002b;

Newsholme et al., 2003a; O'Dowd and Newsholme, 1997; Pithon-Curi et al., 2004). Glutamine in particular also plays a key role in supporting glutathione synthesis (Denno et al., 1996; Newsholme et al., 2003a and b; Roth et al., 2002). Similarly, arginine has been shown to play an important role in PMN-mediated immune functions and has received interest as an agent to promote antimicrobial as well as antitumor effector systems. However, the mechanism by which arginine stimulates or modulates the immune system still remains unclear (Evoy et al., 1998; Moffat et al., 1996; Moinard et al., 1999 and 2000; Mulligan et al., 1998; Schapira et al., 1998). Although its mode of action is not fully understood, arginine is known to be involved in the secretion of anabolic hormones (e.g. growth hormone, insulin, etc.) and the synthesis of key metabolites such as ornithine and citrulline, and also glutamate, aspartate, alanine, proline and nitric oxide (Bansal and Ochoa, 2003; Barbul, 1990; Mühling et al., 2004 and 2002b; Singh et al., 2002; Suchner et al., 2000). Interestingly, arginine (via ornithine) is also employed for polyamine synthesis, a process that plays a key role in cellular proliferation for both normal and tumor cells (Satriano, 2004; Stechmiller et al., 2004; Walters et al., 1992). Moreover, interesting findings suggest that taurine appears to play a pivotal role in important physiological leukocyte immune functions and may have beneficial pharmacological value in modulating cellular metabolism as well (Schaffer et al., 2003; Schuller-Levis and Park, 2004 and 2003). In PMN, taurine accounts for approximately 60% of all free intracellular amino acids and as a result of various studies it became clear that taurine, although once regarded as an inert amino acid, fulfills the criteria for a potent molecule that regulates the dynamic PMN free amino acid pool (i.e. neutral amino acids) and modulates PMN host defense mechanisms and immunoregulation (Mühling et al., 2002a and b; Stapleton et al., 1998a and 1997).

Summing up: with our current state of knowledge, no information is available which describes and compares the precise effects of L-alanyl-L-glutamine, arginine or taurine on PMN free α-keto-acid profiles as well as on essential PMN immune functions, although important underlying pathophysiological aspects still remain unclear. Moreover, our current understanding of the rate of L-alanyl-L-glutamine, arginine or taurine utilization raises some intriguing questions about possible therapeutic manipulation and whether the immunological competence of PMN can be beneficially altered or not.

The goals of this study were therefore:

1) To document the effects of L-alanyl-L-glutamine, arginine, or taurine (regarding its role in PMN immunonutrition) on PMN free intracellular α-keto-acid concentrations.
2) To investigate the effects of L-alanyl-L-glutamine, arginine, or taurine on the activity of released myeloperoxidase, as well as on superoxide anion and hydrogen peroxide formation (as markers of PMN function) in order to show possible parallels with changes in PMN α-keto-acid concentrations.
3) To examine whether a critical dose as well as duration of exposure is required to produce any significant effects.

Materials and methods

The study was approved the local ethical committee of the Justus Liebig University, Giessen. Ten men between 26 and 40 years (33 ± 4.8) with an average height of 179.0 cm (range 171–188) and weight of 79.4 kg (range 73–90) were selected. Those men with metabolic (e.g. diabetes, etc.), cardiopulmonary, neurological or allergic diseases or men taking drugs were excluded. Whole blood samples (lithium-heparinate plastic tubes) were withdrawn between 08:00 and 09:00 (after 10 hours of fasting) with consideration of circadian variations.

L-alanyl-L-glutamine, arginine, taurine

1) To document any dose-dependent effects PMN were incubated with different L-alanyl-L-glutamine (0.25, 0.5, 1, 2.5 and 5 mM), arginine (0.05, 0.1, 0.2, 0.5 and 1 mM) or taurine concentrations (0.05, 0.1, 0.2, 0.5 and 1 mM) for 120 min. The selected L-alanyl-L-glutamine, arginine or taurine concentration corresponded to 1/2-, 1-, 2-, 5- and 10-fold the clinically achieved plasma concentrations (see Bender (1985) for physiological values).
2) To examine if there is a critical duration of exposure necessary to produce any significant effects PMN were incubated with L-alanyl-L-glutamine (5 mM), arginine (1 mM) or taurine (1 mM) for 10, 30, 60 or 120 min.

Solutions of L-alanyl-L-glutamine, arginine or taurine were prepared and diluted in Hank's balanced salt solution (HBSS; Sigma, Deisenhofen, Germany), and the pH in the test solution was confirmed to be 7.4. One milliliter of whole blood was incubated with 25 μl of test solution (final concentrations were as described above) at 37°C using a vibrating water bath. Corresponding volumes of HBSS were added to the control tubes. Before further processing all fractions were immediately cooled in an ice water bath at 4°C and 100 μg/ml phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin, as well as 10 μg/ml antipain (all acquired from Sigma, USA) were added to each plastic heparin tube before the blood samples; these additions served to inhibit proteases.

Highly selective separation of polymorphonuclear leucocytes (PMN) from whole blood

Precise details of our PMN-separation technique have been described previously (Mühling et al., 2004, 2003, 2002a and b). This method is a further development of the methods described by Eggleton et al. (1989)

and Krumholz et al. (1995 and 1993) which allows a very rapid and selective enrichment of PMN from very small quantities of whole blood while preserving high cellular viability and integrity.

Chromatographic amino and α-keto acid analysis

α-keto acids in PMN were quantified using previously described methods which fulfill the strict criteria required for ultrasensitive, comprehensive amino- and α-keto acid analysis, and which were especially developed and precisely validated in our institute for this purpose (for details see Mühling et al., 2003). The coefficients of variations for both the method reproducibility and retention time reproducibility were within the normal range. PMN α-keto acid concentrations are given in 10^{-17} mol per PMN-cell (Fig. 1).

Superoxide anion (O_2^-), hydrogen peroxide production (H_2O_2) and activity of released myeloperoxidase (MPO) were determined photometrically using modifications of known methods validated in our institute for this purpose (Krumholz et al., 1995 and 1993).

Superoxide anion production was measured by reduction of cytochrome C. Cytochrome C (100 mg, type IV, Sigma, Deisenhofen, Germany) was dissolved in 30 ml PBS® glucose buffer (Gibco, Karlsruhe, Germany). The solution was portioned and stored at −20°C. Opsonized zymosan (Sigma, Deisenhofen, Germany) was used to stimulate PMN. It was evoked 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 stored at −20°C. Whole blood was incubated either with L-alanyl-L-glutamine, arginine or taurine. These preparations were then incubated for 10, 30, 60 or 120 min at 37°C (vibrating water bath). The PMN were thereafter isolated using a modified 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 administration of 7 ml PBS® stock buffer, the tubes were 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 (0.8×10^6/ml) was adjusted by adding PBS® containing 9.99 g/l glucose (Merck, Darmstadt, Germany). After PMN isolation, 500 μl zymosan, 150 μl pool serum, 250 μl cytochrome C and 500 μl isolated PMN suspension (0.8×10^6/ml) and again L-alanyl-L-glutamine, arginine or taurine were poured into a test tube. A preparation containing 500 μl buffer instead of zymosan was used for zero adjustment. After incubation for 15 min at 37°C, the reaction was stopped by putting the test tube into iced water. After centrifugation (350 × g; 3 min, 4°C) extinction of the supernatant was measured photometrically (546 nm; Digitalphotometer 6114S®; Eppendorf, Germany). The amount of superoxide anions measured resulted from the extinction coefficient of cytochrome C (Rick, 1977). All control probes were prepared, incubated and measured in the same way.

Hydrogen peroxide production was also determined photometrically using a method based on horseradish peroxidase catalyzed oxidation of phenol red by hydrogen peroxide. Phenol red (Sigma, Deisenhofen, Germany) and horseradish peroxidase (Type II, Sigma, Deisenhofen, Germany) were added to PMN which had been stimulated by opsonized zymosan. Phenol red was dissolved in double-distilled water (10 g/l). Horseradish peroxidase was dissolved in PBS® glucose buffer (5 g/l). After incubation of whole blood with either L-alanyl-L-glutamine, arginine or taurine for 10, 30, 60 or 120 min at 37°C, PMN were isolated as described above. Isolated PMN were stimulated by opsonized zymosan. The final preparation consisted of 500 μl zymosan, 125 μl pool serum, 12.5 μl horseradish peroxidase, 12.5 μl phenol red, 12.5 μl sodium azide (200 mmol/l; Merck, Darmstadt, Germany), 500 μl PMN suspension (2×10^6 PMN cells/ml) and again arginine, L-alanyl-L-glutamine or taurine (15 min, 37°C). After adding of 25 μl 1N sodium hydroxide solution (Merck, Darmstadt, Germany), the extinction was measured photometrically at 623 nm. All control probes were prepared, incubated and measured in the same way.

Activity of released myeloperoxidase: 1 mmol/l 2,2′-azino-di-(3-ethylbenzthiazoline) sulfonic acid (ABTS, Sigma, Deisenhofen, Germany) was dissolved in 0.1 mol citrate buffer (Behring, Marburg, Germany; pH 7.4). The preparation of whole blood was incubated either with L-alanyl-L-glutamine, arginine or taurine for 10, 30, 60 or 120 min at 37°C. After this incubation, 100 μl isolated PMN suspension (2×10^6/ml) was once more incubated with 0.5 μg cytochalasin B (Sigma, Deisenhofen, Germany) and again with L-alanyl-L-glutamine, arginine or taurine for 5 min (37°C). After addition of 100 μl opsonized zymosan and supplementation of L-alanyl-L-glutamine, arginine or taurine in order to keep the concentration constant, the preparation was incubated again for 10 min (37°C). Then 1 ml ABTS solution was added. After centrifugation (700 × g, 5 min, 20°C), 1 ml supernatant was removed and mixed with 1 μl hydroxide peroxide solution (30%; Merck, Darmstadt, Germany) and the extinction coefficient was measured at 405 nm.

Statistical analysis

Statistical analysis and interpretation of the results were performed in close cooperation with colleagues from the Department of Medical Statistics, Justus Liebig University Giessen.

All tests were performed in duplicate. Thus our PMN amino acid results are the mean of two estimations. After the results were demonstrated to be normally distributed (Pearson-Stephens test), statistical methods were performed including Bartlett's test to check homogeneity of variance ($p \leq 0.1$). If the requirements were met, ANOVA analysis was conducted. If the requirements were not fulfilled, the Friedman test was performed. Probability levels of $p \leq 0.05$ versus control were considered significant. The data are given as arithmetic means ± standard deviations (mean ± SD).

Results

The free intracellular α-keto acid concentrations (Fig. 1), superoxide anion formation, hydrogen peroxide generation as well as activity of released myeloperoxidase obtained in the control cells were within normal physiological ranges [for details see (Krumholz et al., 1995 and 1993; Mühling et al., 2004 and 2003)].

Effects of L-alanyl-L-glutamine on free α-keto acid pool in PMN

Following low L-alanyl-L-glutamine doses (0.25 mM) concentrations of free intracellular α-keto acids remained unaffected (Table 1). In the presence of ≥0.5 mM L-alanyl-L-glutamine, significant increases in PMN α-ketoglutarate and pyruvate were observed (PMN incubation for 120 min; Table 1). Relevant changes in PMN α-ketoglutarate and pyruvate concentrations mainly occurred with PMN incubation for 30 min or longer (5 mM L-alanyl-L-glutamine; Table 2). PMN free α-ketobutyrate, α-ketoisovaleriate, α-ketoisocapronate,

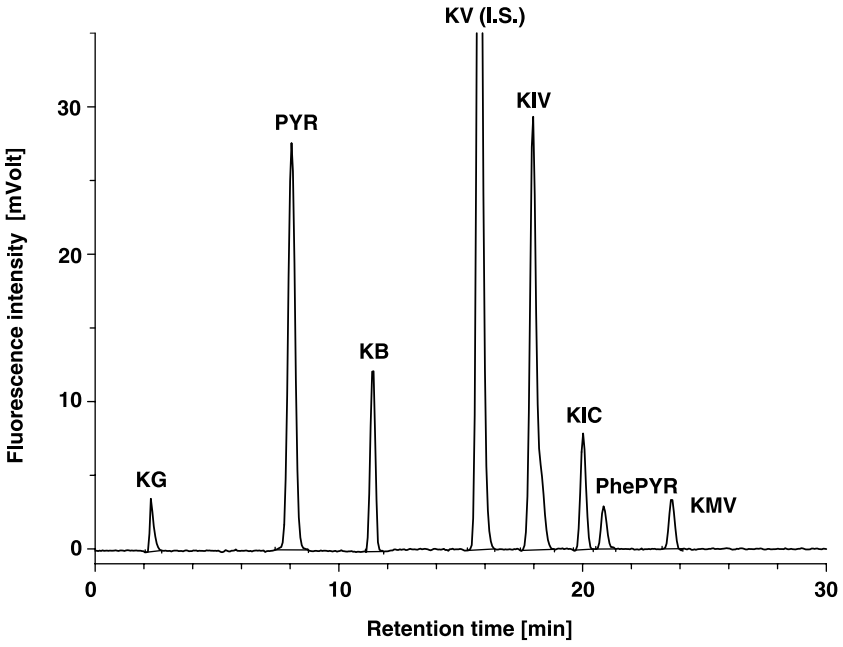

Fig. 1. Typical elution profile of an *PMN sample*. *KG*, α-ketoglutarate; *PYR*, pyruvate; *KB*, α-ketobutyrate; *KIV*, α-ketoisovaleriate; *KIC*, α-ketoisocapronate; *PhePYR*, p-hydroxy-phenylpyruvate; *KMV*, α-keto-β-methylvaleriate; *KV*, α-ketovaleriate (I.S. = internal standard)

Table 1. Free intracellular *α-ketoglutarate*, *pyruvate* and *α-ketobutyrate* concentrations in PMN-cells following *L-alanyl-L-glutamine* (0.25, 0.5, 1, 2.5 and 5 mM; 120 min), *arginine* (0.05, 0.1, 0.2, 0.5 and 1 mM; 120 min) and *taurine* incubation (0.05, 0.1, 0.2, 0.5 and 1 mM; 120 min) of whole blood *in vitro*. PMN amino acid concentrations are given in 10^{-17} Mol per PMN-cell (mean $\pm$ SD; n = 10)

α-Keto acids	*L-Alanyl-L-glutamine/Arginine/Taurine*					
Ala-Gln	*Control*	*0.25 mM*	*0.5 mM*	*1 mM*	*2.5 mM*	*5 mM*
α-KG	1.61 ± 0.38	1.78 ± 0.44	$2.12 \pm 0.49^*$	$2.55 \pm 0.67^*$	$3.01 \pm 0.69^*$	$3.88 \pm 1.06^*$
PYR	6.24 ± 1.19	7.17 ± 1.55	$8.73 \pm 2.18^*$	$10.56 \pm 2.39^*$	$12.25 \pm 3.54^*$	$16.13 \pm 4.12^*$
Arg	*Control*	*0.05 mM*	*0.1 mM*	*0.2 mM*	*0.5 mM*	*1 mM*
α-KG	1.46 ± 0.31	1.39 ± 0.34	1.53 ± 0.30	1.75 ± 0.43	$1.89 \pm 0.42^*$	$2.09 \pm 0.57^*$
PYR	5.98 ± 1.13	5.80 ± 1.27	6.15 ± 1.61	6.68 ± 1.59	$7.47 \pm 1.64^*$	$8.02 \pm 2.11^*$
Tau	*Control*	*0.05 mM*	*0.1 mM*	*0.2 mM*	*0.5 mM*	*1 mM*
PYR	6.03 ± 1.20	6.25 ± 1.43	5.71 ± 1.38	5.37 ± 1.44	$4.97 \pm 1.17^*$	$4.21 \pm 1.12^*$
α-KB	4.67 ± 1.11	4.82 ± 0.99	4.10 ± 1.06	3.73 ± 0.98	$3.19 \pm 0.94^*$	$2.06 \pm 0.59^*$

* $p \leq 0.05$ versus control values

p-hydroxy-phenylpyruvate and α-keto-β-methylvaleriate profiles remained unaltered.

Effects of L-alanyl-L-glutamine on oxidative response and myeloperoxidase activity

All PMN immune functions tested were unaffected up to 0.025 mM L-alanyl-L-glutamine (Table 3). In the presence of higher L-alanyl-L-glutamine supplementation ($\geq$0.5 mM; 120 min), superoxide anion and hydrogen peroxide generation increased significantly (Table 3). Moreover, L-alanyl-L-glutamine ($\geq$1 mM; 120 min) caused significant increases in MPO activity (Table 3). Relevant changes in PMN immune functions tested mainly occurred with PMN incubation for 30 min or longer (5 mM L-alanyl-L-glutamine; Table 4).

Effects of arginine on free α-keto acid pool in PMN

Following low arginine doses ($\leq$0.2 mM) concentrations of free intracellular α-keto acids remained unaffected (Table 1). In the presence of $\geq$0.5 mM arginine, significant increases in PMN α-ketoglutarate and pyruvate were

Table 2. Effects of *L-alanyl-L-glutamine* (5 mM), *arginine* (1 mM) and *taurine* (1 mM) incubated with whole blood for 10, 30, 60 and 120 min on free intracellular *α-ketoglutarate*, *pyruvate* and *α-ketobutyrate* concentrations in PMN (10^{-17} Mol per PMN-cell; mean ± SD; n = 10)

α-Keto acids	*L-Alanyl-L-glutamine/Arginine/Taurine*			
	10 min	*30 min*	*60 min*	*120 min*
Control				
α-KG	1.31 ± 0.29	1.62 ± 0.30	1.38 ± 0.43	1.19 ± 0.32
PYR	6.52 ± 1.63	5.94 ± 1.59	6.17 ± 1.31	5.75 ± 1.36
α-KB	4.40 ± 1.04	4.63 ± 0.96	4.29 ± 1.14	4.16 ± 1.09
Ala-Gln (5 mM)				
α-KG	1.49 ± 0.38	2.09 ± 0.47*,#	2.33 ± 0.54*,#	2.85 ± 0.62*,#
PYR	6.84 ± 1.47	9.12 ± 2.21*,#	10.71 ± 3.09*,#	12.49 ± 3.60*,#
α-KB	4.87 ± 1.32	4.35 ± 0.93	4.56 ± 1.22	4.28 ± 0.97
Arg (1 mM)				
α-KG	1.26 ± 0.35	1.45 ± 0.42	1.71 ± 0.34*,#	1.94 ± 0.48*,#
PYR	6.34 ± 1.66	6.81 ± 1.75	7.58 ± 1.45*	7.92 ± 1.61*,#
α-KB	4.53 ± 1.04	4.24 ± 0.80	4.96 ± 1.37	4.39 ± 1.18
Tau (1 mM)				
α-KG	1.25 ± 0.29	1.43 ± 0.31	1.55 ± 0.42	1.30 ± 0.37
PYR	5.98 ± 1.17	5.36 ± 1.40	4.81 ± 1.02*,#	4.19 ± 0.30*,#
α-KB	4.33 ± 0.95	3.57 ± 0.83*	2.64 ± 0.30*,#	2.02 ± 0.58*,#

* $p \leq 0.05$ versus control values; # $p \leq 0.05$ 120 min versus 10 min

Table 3. Effects of different *L-alanyl-L-glutamine* (0.25, 0.5, 1, 2.5 and 5 mM), *arginine* (0.05, 0.1, 0.2, 0.5 and 1 mM) and *taurine* concentrations (0.25, 0.05, 0.1, 0.25 and 0.5 mM) incubated with whole blood for 120 min on PMN *superoxide anion production* [fmol/(PMN • min)$^{-1}$], *hydrogen peroxide formation* [fmol/(PMN • min)$^{-1}$] and *myeloperoxidase activity* [units/l supernatant]; (mean ± SD; n = 10)

O_2^-, H_2O_2, MPO	*L-Alanyl-L-glutamine/Arginine/Taurine*					
Ala-Gln	*Control*	*0.25 mM*	*0.5 mM*	*1 mM*	*2.5 mM*	*5 mM*
O_2^-	3.127 ± 0.451	3.348 ± 0.468	3.589 ± 0.532*	3.877 ± 0.566*	5.065 ± 0.886*	7.088 ± 1.516*
H_2O_2	1.141 ± 0.258	1.192 ± 0.283	1.478 ± 0.335*	1.631 ± 0.376*	2.334 ± 0.592*	2.941 ± 0.802*
MPO	0.412 ± 0.122	0.461 ± 0.133	0.498 ± 0.149	0.604 ± 0.180*	0.719 ± 0.221*	1.002 ± 0.318*
Arg	*Control*	*0.05 mM*	*0.1 mM*	*0.2 mM*	*0.5 mM*	*1 mM*
O_2^-	3.265 ± 0.478	3.011 ± 0.429	2.732 ± 0.403*	2.387 ± 0.355*	2.019 ± 0.312*	1.206 ± 0.212*
H_2O_2	1.248 ± 0.282	1.395 ± 0.302	1.527 ± 0.343	1.497 ± 0.258*	1.714 ± 0.399*	1.907 ± 0.559*
MPO	0.439 ± 0.127	0.439 ± 0.127	0.503 ± 0.148	0.548 ± 0.161	0.617 ± 0.176*	0.701 ± 0.212*
Tau	*Control*	*0.05 mM*	*0.1 mM*	*0.2 mM*	*0.5 mM*	*1 mM*
O_2^-	3.321 ± 0.449	3.286 ± 0.453	3.120 ± 0.439	2.462 ± 0.393*	2.108 ± 0.368*	1.527 ± 0.293*
H_2O_2	1.303 ± 0.251	1.303 ± 0.251	1.303 ± 0.251	0.940 ± 0.219*	0.787 ± 0.192*	0.551 ± 0.140*
MPO	0.454 ± 0.136	0.482 ± 0.151	0.531 ± 0.160	0.612 ± 0.189	0.647 ± 0.193*	0.715 ± 0.224*

* $p \leq 0.05$ versus control values

observed (PMN incubation for 120 min; Table 1). Relevant changes in PMN α-ketoglutarate and pyruvate concentrations mainly occurred with PMN incubation for 60 min or longer (1 mM arginine; Table 2). PMN free α-ketobutyrate, α-ketoisovaleriate, α-ketoisocapronate, p-hydroxy-phenylpyruvate and α-keto-β-methylvaleriate profiles remained unaffected.

Effects of arginine on oxidative response and myeloperoxidase activity

All PMN immune functions tested were unaltered up to 0.05 mM arginine (Table 3). In the presence of higher arginine supplementation (≥0.1 mM; 120 min), superoxide anion generation decreased significantly (Table 3). Moreover, arginine caused significant increases in hydro-

Table 4. Effects of *L-alanyl-L-glutamine* (5 mM), *arginine* (1 mM) and *taurine* (1 mM) incubated with whole blood for 10, 30, 60 and 120 min on PMN *superoxide anion production* [fmol/(PMN • min)$^{-1}$], *hydrogen peroxide formation* [fmol/(PMN • min) $^{-1}$] and *myeloperoxidase activity* [units/l supernatant]; (mean ± SD; n = 10)

O_2^-, H_2O_2, *MPO*	*L-Alanyl-L-glutamine/Arginine/Taurine*			
	10 min	*30 min*	*60 min*	*120 min*
Control				
O_2^-	3.298 ± 0.501	3.316 ± 0.532	3.226 ± 0.551	3.047 ± 0.484
H_2O_2	1.187 ± 0.261	1.128 ± 0.247	1.165 ± 0.270	1.058 ± 0.245
MPO	0.543 ± 0.161	0.567 ± 0.176	0.529 ± 0.158	0.485 ± 0.153
Ala-Gln (5 mM)				
O_2^-	3.917 ± 0.636	4.613 ± 0.784$^{*,\#}$	5.227 ± 0.849$^{*,\#}$	6.648 ± 1.460$^{*,\#}$
H_2O_2	1.311 ± 0.298	1.782 ± 0.365$^{*,\#}$	2.191 ± 0.590$^{*,\#}$	2.722 ± 0.643$^{*,\#}$
MPO	0.606 ± 0.174	0.798 ± 0.201$^{*,\#}$	0.841 ± 0.240$^{*,\#}$	1.329 ± 0.433$^{*,\#}$
Arg (1 mM)				
O_2^-	2.806 ± 0.443	2.146 ± 0.358$^{*,\#}$	1.458 ± 0.234$^{*,\#}$	0.925 ± 0.153$^{*,\#}$
H_2O_2	1.191 ± 0.260	1.308 ± 0.296	1.492 ± 0.281$^{*,\#}$	1.667 ± 0.398$^{*,\#}$
MPO	0.529 ± 0.166	0.658 ± 0.197	0.708 ± 0.191*	0.756 ± 0.217$^{*,\#}$
Tau (1 mM)				
O_2^-	2.997 ± 0.489	2.707 ± 0.467*	2.139 ± 0.343$^{*,\#}$	1.603 ± 0.264$^{*,\#}$
H_2O_2	1.016 ± 0.231	0.876 ± 0.192*	0.699 ± 0.159$^{*,\#}$	0.486 ± 0.114$^{*,\#}$
MPO	0.527 ± 0.161	0.688 ± 0.213	0.753 ± 0.238	0.786 ± 0.250$^{*,\#}$

* $p \leq 0.05$ versus control values; $^{\#}$ $p \leq 0.05$ 120 min versus 10 min

gen peroxide formation ($\geq$0.2 mM; 120 min; Table 3) and MPO activity ($\geq$0.5 mM; 120 min; Table 3). Relevant changes in the PMN immune functions tested occurred mainly with PMN incubation for 30 min or longer (1 mM arginine; Table 4).

Effects of taurine on free α-keto acid pool in PMN

Following low taurine doses ($\leq$0.2 mM) concentrations of free intracellular α-keto acids remained unaffected (Table 1). In the presence of $\geq$0.5 mM taurine, significant decreases in PMN free pyruvate and α-ketobutyrate were obeserved (PMN incubation for 120 min; Table 1). Relevant changes in PMN pyruvate and α-ketobutyrate concentrations occurred mainly with PMN incubation for 60 min or longer (1 mM taurine; Table 2). PMN free α-ketoglutarate, α-ketoisovaleriate, α-ketoisocapronate, p-hydroxy-phenylpyruvate and α-keto-β-methylvaleriate profiles remained unaffected.

Effects of taurine on oxidative response and myeloperoxidase activity

All PMN immune functions tested were unaffected up to 0.1 mM taurine (Table 3). In the presence of higher arginine supplementation ($\geq$0.2 mM; 120 min), superoxide anion generation and hydrogen peroxide formation decreased significantly (Table 3). Moreover, taurine caused significant increases in MPO activity ($\geq$0.5 mM; 120 min; Table 3). Relevant changes in PMN immune functions occurred usually with PMN incubation for 30 min or longer (1 mM taurine; Table 4).

Discussion

Exogenous L-alanyl-L-glutamine

L-alanyl-L-glutamine supplementation to whole blood significantly increased free intracellular α-ketoglutarate as well as pyruvate levels in PMN. Other α-keto-acids examined in this study remained uninfluenced. Interestingly, our own earlier findings revealed that L-alanyl-L-glutamine supplementation to whole blood significantly increased free intracellular glutamine as well as alanine profiles, and that they also altered other important PMN free intracellular amino acid profiles (i.e. glutamate, aspartate, asparagine, ornithine, arginine, serine and glycine), a fact that indicated an increased uptake of alanine and glutamine by PMN from plasma and/or an increased uptake of L-alanyl-L-glutamine dipeptide before intracellular hydrolysis and further utilization (Mühling et al., 2002b). Indeed, high plasma L-alanyl-L-glutamine hydrolase activity before uptake of the liberated amino acid residues has been described elsewhere (Hubl et al.,

1989). Moreover, various tissue and blood cells contain sufficient membrane bound as well as intracellular hydrolase activity (Albers et al., 1988; Fürst et al., 1997a; Stehle et al., 1991 and 1989). As such, L-alanyl-L-glutamine dipeptides in particular have been found to be a suitable source for glutamine and alanine that have comparable biological activity (Fürst et al., 1997a and b, 1993, 1990a and b). In the author's opinion, our findings would favor the latter hypothesis since high rates of glutamine as well as alanine uptake and utilization, especially by rapidly dividing cells such as PMN, have been described elsewhere (Curi et al., 1997a and b; Newsholme et al., 2003a and b, 2001, 1987; Pithon-Curi et al., 2004, 2002a and b). From the results obtained until now, glutamine like glucose as acts as substrate for subsequent intracellular metabolization, and this process also occurs within PMN cells (Curi et al., 1999; Newsholme et al., 1985; Pithon-Curi et al., 2004). Enzymes required for this (e.g. glutaminase, aspartate aminotransferase, alanine aminotransferase) are certainly present in PMN cells as others have shown (Fauth et al., 1993 and 1990; Moinard et al., 2002a). The demand-controlled intracellular conversion of the intragranulocytic carbon and nitrogen store glutamine to glutamate is of special metabolic relevance. The enzyme catalyzing this, a phosphate dependent glutaminase, is highly active in PMN cells ($\approx$56 nmol/min/mg protein) and can be significantly influenced by extra and intracellular stimuli (e.g. proinflammatory mediators) (Curi et al., 1997a and b; Garcia et al., 1999). The glutamate arising from this reaction plays a key metabolic role since it can be very rapidly transformed into other essential cell substrates as a so-called ''intracellular turntable'' for amino nitrogen metabolism. Reversible transaminase reactions (the equilibrium constant in leukocytes as in other cells lies at approximately 1 that release NH_3 deserve special mention here (Belfiore et al., 1975; Luciak and Trznadel, 1983; Mizuho et al., 1996; Stjernholm et al., 1969). According to Washizu et al. (1998), oxidative deamination of glutamate can also lead to the formation of α-ketoutarate. The carbon backbone arising from glutamine oxidation can be shunted directly into the tricarboxylic acid cycle in the form of α-ketoglutarate. In this way glutamine is also indirectly available as a substrate for the enzymes of the urea cycle (e.g. in the form of aspartate via oxalacetate) (Curi et al., 1997a and b; Newsholme et al., 2003a and b, 2001, 1987; Wolfe and Gatfield, 1975). Thus regulation of glutamine metabolism, the formation of arginine and ornithine, and ultimately (with PMN activation) the synthesis of $\bullet$NO can all be modulated (Moinard et al., 2002b; Mühling et al., 2002b; O'Dowd and Newsholme, 1997). The conversion of malate to oxaloacetate by the $NADP^+$-dependent malate dehydrogenase is particularly important for the metabolization of α-ketoglutarate by the enzymes of the TCA cycle (Newsholme et al., 1987; Stjernholm et al., 1969). Ultimately, glutamine can be converted to pyruvate, the starting point for the ''de novo synthesis'' of glucose from α-ketoglutarate (Agam and Gutman, 1972; Curi et al., 1986; Mitzkat et al., 1972; Newsholme et al., 1987; Willems et al., 1978). The metabolization of glutamine by the TCA cycle does not just supply intracellular carbon and nitrogen precursors for the above-mentioned metabolic pathways, since it also provides NADPH (Ardawi and Newsholme, 1982; Board et al., 1990; Brand et al., 1989; Frei et al., 1975). NADPH can arise from the activities of isocitrate, α-ketoglutarate and malate dehydrogenase as well as from the conversion of pyruvate by pyruvate dehydrogenase. NADPH plays a special and essential role in PMN cells because of its further metabolism by the membranous NADPH oxidase. The activation of this enzyme complex provides superoxide anions and in so doing forms an essential component of the granulocytic immune defense (Burg and Pillinger, 2001; Dahlgren and Karlsson, 1999; Frei et al., 1975; Kobayashi et al., 2001). An increase in glutamine metabolism due to an inflammatory event does not just lead to essential metabolic precursors, but much more significantly it increases the participation of the immediately available intragranulocytic redox systems. For this reason it is not surprising that the increase in intragranulocytic glutamine and alanine occurring upon application of L-alanyl-L-glutamine is also associated with a large increase in extragranulocytically produced superoxide anion and hydrogen peroxide as well as a clear increase in extracellular myeloperoxidase activity.

Exogenous arginine

Exogenous arginine added to whole blood significantly increased PMN intracellular α-ketoglutarate as well as pyruvate content (requiring $\geq$60 min exposure at the highest concentration) without influencing any other α-keto acid. Moreover, and concerning our own earlier findings, we demonstrated reproducibly that arginine incubated in whole blood significantly increased free intracellular arginine, the basic amino acids ornithine and citrulline as well as glutamate, aspartate and alanine, a fact that draws attention to arginine's role as an important intracellular substrate for various amino acid pathways (Mühling et al.,

2002b). This is not surprising considering that arginine, analogously to ornithine, can enter various immune cells via a number of active transport mechanisms (i.e. sodium-coupled or diffusion), although our study does not allow us to infer whether the effects were direct (as a result of direct PMN intracellular arginine conversion into amino and α-keto-acid metabolites) or due to metabolic changes induced by arginine incubation of whole blood (i.e. uptake of arginine and amino and α-keto-acids by PMN produced from other blood cells (Bansal and Ochoa, 2003; Calder, 2003; Fürst, 2000; Grimble et al., 2001 and 1998; Montejo et al., 2003). The pattern of PMN α-keto acid changes following arginine incubation, however, favors the hypothesis that increases in PMN free arginine concentrations are followed by arginine conversion into amino acid derivatives (i.e. α-ketoglutarate and pyruvate) (Barbul, 1990; Evoy et al., 1998; Moinard et al., 2000; Suchner et al., 2000). Regarding these findings we suggest an anabolic effect of arginine added to whole blood *in vitro* due to an increase in PMN α-keto and amino acids that are important for providing nitrogen and carbon for the synthesis of macromolecules. Such α-keto and amino acids also act as oxidative fuels for energy production because of their entry into the tricarboxylic acid cycle via aspartate and glutamate, substrates that are also elevated following arginine supplementation (Bihari, 2002; Blanc et al., 2005; Fürst, 2000; Moinard et al., 2002b; Mühling et al., 2004; Singh et al., 2002).

Moreover, regarding PMN immune functions arginine incubation of PMN significantly decreased superoxide anion formation as well as distinctly increased both hydrogen peroxide generation and myeloperoxidase activity. This interesting interplay between exogenous arginine and PMN reactive oxygen species (ROS) as well as enzymes produced need further explanation. Various authors suggest that arginine (for example via ornithine), since arginase has been isolated to a relatively high amount, may enhance leukocyte immune function and improves the host's capacity to resist infection (Bansal and Ochoa, 2003; Grimble et al., 2001 and 1998; Munder et al., 2005; Schapira et al., 1998). Indeed, several essential antimicrobial functions of human leukocytes (i.e. nitric oxide synthase dependent pathways) greatly depend on intracellular arginine and its conversion into immunocompetent molecules (Blantz and Munger, 2002; Cedergren et al., 2003). Moreover, a combination of PMN free aspartate, ornithine, arginine, glutamate and glutamine profiles in particular have been found to be highly predictive regarding the levels of PMN energy charge and protein synthesis (Metcoff et al., 1989, 1988, 1987 and 1986). Because of this we suggest here that nutritional or pharmacological regimes that enhance the supply of arginine to PMN may be of considerable value for modulating essential PMN functions. Moreover, regarding hydrogen peroxide and superoxide anion formation, our results suggest paradoxically that arginine supplementation can both augment and inhibit free radical generation in human PMN. These findings confirm previous studies which postulated that increases in PMN free intracellular arginine (e.g. metabolized from ornithine) inhibit free superoxide anion generation as suggested by the inverse correlation between PMN arginine derived •NO formation and superoxide anion generation (Kausalya and Nath, 1998; Rodenas et al., 1998 and 1996; Salvemini et al., 2003; Seth et al., 1994; Wiedermann et al., 1993). We suggest that dietary arginine supplementation should receive new attention because of its role in modulating the PMN α-keto and amino acid pools and essential immune functions. However, further research is necessary to clarify arginine's *in vivo* therapeutic immunoregulatory properties.

Exogenous taurine

Taurine supplementation to whole blood significantly decreased PMN intracellular pyruvate and α-ketobutyrate content. Other α-keto acids examined in this study were not altered. Previous studies by our group (Mühling et al., 2002a and b) showed that high exogenous taurine concentrations (1 mM) incubated in whole blood increased PMN taurine levels by approximately 27%, an unsurprising fact considering that taurine can enter immune cells both via active sodium-coupled transport or diffusion (as described for other cell types) (Jacobson et al., 1986; Porter et al., 1991). Moreover, as intracellular taurine increases, the concentrations of the small neutral amino acids (serine, glycine, threonine and alanine) were significantly reduced too, an effect which might also be involved in reductions observed in their transamination or deamination products (i.e. pyruvate and α-ketobutyrate) (Mühling et al., 2002b). Summing up: our findings, again, may draw attention to taurine's role as an important intracellular volume-regulating osmolyte. Indeed, for example Chen and Kempson (1995), Cuisinier et al. (2002) as well as Schaffer et al. (2000) all stated that taurine, as well as neutral amino acids, may function as volume regulating osmolytes and that corresponding decreases in neutral amino acids following increases in PMN taurine levels may suggest that recompartmentalization of amino acids occurred following taurine treatment so that the osmotic balance was

retained to some extent. However, the decrease in pyruvate and α-ketobutyrate content and total neutral amino acid concentrations (Mühling et al., 2002b) only partially ($\approx$60%) compensated for the changes in PMN free amino acid content brought about by the raised intracellular taurine levels.

Apart from osmoregulation, extracellular as well as intracellular taurine in particular may act as an antioxidant by preventing oxidative damage, by protecting against attack from chlorinated oxidants (i.e. HOCl) and by inactivating important enzymes (i.e. MPO) (Schuller-Levis and Park, 2004 and 2003). In this study, taurine suppressed superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) formation and increased released myeloperoxidase (MPO) activity both in a dose- and duration of exposure-dependent manner. These results confirm earlier studies as well as clinical findings, but they do need to be looked into further (Masuda et al., 1984a and b; McLoughlin et al., 1991; Raschke et al., 1995; Redmond et al., 1998). Paradoxically, the PMN $O_2^-/H_2O_2/MPO/Cl^-$ system is known to be both beneficial and detrimental to the host organism since the production of reactive oxygen species (ROS) and HOCl in particular is required for intra- as well as extracellular bacterial killing. Activation of neutrophils results in an immense production of O_2^- and H_2O_2, however ROS- and HOCl-toxicity is not just restricted to bacterial membranes since additional host cell, tissue and epithelial damage can also be induced after inflammatory cell activation (Redmond et al., 1998; Stapleton et al., 1998a). ROS scavengers (i.e. taurine) are known to attenuate the deleterious effects of these toxic intermediates (Raschke et al., 1995; Schuller-Levis and Park, 2004 and 2003). Earlier findings suggest that the protective activity of taurine may reside in its ability to become chlorinated in the presence of chlorinated oxidants (e.g. HOCl), thereby preventing the direct intracellular (i.e. mitochondria) as well as extracellular attack (PMN, blood cells and also tissue cells) of this oxidant on cell membranes (Farriol et al., 2002; Masuda et al., 1984a and b; McLoughlin et al., 1991; Schuller-Levis et al., 1992 and 1990). The authors therefore suggest that both endogenous taurine and taurine substitution may act as antioxidants by complexing MPO-derived HOCl [formed from H_2O_2, which is itself formed from O_2^- via superoxide dismutase and Cl^- with taurine to form taurine-chloramine (Tau-Cl)]. This may limit free radical-induced cytotoxicity while maintaining the PMN's microbicidal capability, and in this way confer dual biological benefits. Moreover, consistent with former studies, we hypothesize that taurine strongly regulates both intracellular and extracellular myeloperoxidase activity in PMN (Marquez and Dunford, 1994; Masuda et al., 1984a and b; Son et al., 1998; Stapleton et al., 1998a and b, 1997). From our results we therefore believe that the decreased O_2^-- and H_2O_2 formation may be due to the following: either 1) a reduction of HOCl-mediated ROS due to sequestration of HOCl by taurine to form taurine chloramine, or 2) an increased O_2^- and H_2O_2 metabolism due to increased MPO activity, or indeed both. Summing up: although much remains to be clarified regarding the complete biochemical and metabolic functions of taurine supplementation, our data confirm that exogenous taurine incubated in whole blood might ameliorate defective phagocytic/pro-inflammatory cell microbicidal capacity and counteract the decreased cell viability associated with PMN aging so that the immunological competence of PMNs is strengthened. Because of this study it is now becoming clear that although once regarded as an inert amino acid, taurine fulfills the necessary criteria as a potent molecule for regulating the dynamic PMN free α-keto and amino acid pools as well as for modulating PMN host defense mechanisms and immunoregulation. These findings therefore contribute further evidence that benefits might indeed arise from including this amino acid in modern therapeutic protocols.

Overall, the effects of L-alanyl-L-glutamine, arginine or taurine on PMN free intracellular α-keto-acids and essential PMN immune functions have now been investigated for the first time. From our results it is clear that L-alanyl-L-glutamine, arginine or taurine fulfills the criteria for potent molecules that regulate the dynamic α-keto-acid pools and that modulate PMN host defense mechanisms and immunoregulation. However, further *in vivo* research is required to clarify L-alanyl-L-glutamine's, arginine's or taurine's role regarding their therapeutic, immunoregulatory or immunomodulatory properties.

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