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Nitric oxide and polyamine pathway-dependent modulation of neutrophil free amino- and α-keto acid profiles or host defense capability

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Summary. We have examined the effects of N_ω-nitro-L-arginine-methyl-ester-hydrochloride [L-NAME; inhibitor of nitric oxide synthase], S-nitroso-N-acetyl-penicillamine [SNAP; nitric oxide donor], α-difluoro-methyl-ornithine [DFMO; inhibitor of ornithine decarboxylase] arginine or ornithine as well as the combination of arginine or ornithine with L-NAME, SNAP or DFMO on intracellular free amino- and α-keto acid profiles and the immune function markers superoxide anion and hydrogen peroxide generation as well as released myeloperoxidase activity in neutrophils (PMN). Although the underlying mechanisms still remain unclear, we believe from our results that nitric oxide as well as polyamine-dependent pathways are involved in the signal transmission of free radical molecule, beneficial nutritional therapy or maleficient pharmacological stress-induced alterations in PMN nutrient composition. Relevant changes in intragranulocyte free amino- and α-keto acid homeostasis and metabolism, especially, may be one of the determinants in PMN nutrition that positively or negatively influences and modulate neutrophil host defence capability and immunocompetence.

Keywords: Nitric oxide – Polyamines – Neutrophil – Amino acids – α-Keto acids – Immune function

Introduction

Regarding arginine and ornithine in PMN, two specific metabolic activities in particular have been associated with the potential immunomodulatory properties of these amino acids: on the one hand there is the so-called ''argininase reaction'', through which urea as well as the basic amino acid ornithine arise after the hydrolytic cleavage of a guanidine group (Bronte et al., 2003; Munder et al., 2005). This important enzyme of the urea cycle also has a very high activity in PMN and serves to provide free ornithine for polyamine biosynthesis in these as well as other cells (Bansal and Ochoa, 2003). The non-proteinogenic ornithine is converted by the enzyme ornithine decarboxylase in various organelles to putrescine, from which the polyamines spermine and spermidine can be formed (Moinard et al., 2002a, b; Walters and Wojcik, 1994). This metabolic pathway in particular appears to be of essential importance for immune cells amongst others, although various studies have shown that polyamine synthesis has an important influence on cellular mitogenesis and the cytotoxicity of lymphocytes and macrophages and presumably also appears to represent an important immunoregulatory metabolic process for PMN (Guarnieri et al., 1987; Mariggio et al., 2004; Kafy et al., 1986; Ratasirayakorn et al., 1999; Walters and Chapman, 1995; Walters et al., 1995, 1998). Another way in which arginine can induce cellular immunomodulation also in PMN cells is through the double NADPH-dependent hydroxylation via N-hydroxyarginine to nitric oxide ($^\bullet$NO), a ubiquitous metabolic pathway that has been detected in many different body cells and especially also in inflammatory cells such as PMN, lymphocytes and macrophages (Efron and Barbul, 2000; Galijasevic et al., 2003; Isenberg, 2003; Murphy and Newsholme, 1996; Raykova et al., 2003; Sethi et al., 2001; Tong and Barbul, 2004; Witte and Barbul, 2003). Through the calcium, calmodulin and tetrahydrobiopterin-dependent enzyme $^\bullet$NO synthase, the colourless gas $^\bullet$NO is formed from arginine upon liberation of citrulline (Bronte and

Zanovello, 2005; Cynober, 2004). The discovery and characterisation of this gas led to the Nobel prize being awarded to Robert F. Furchgott, Felix Murad and Louis Ignarro in 1998 (Salvemini et al., 2003b; Satriano, 2004). In 1986 the above-mentioned authors simultaneously and independently came to the revolutionary finding that the messenger discovered in 1980 and first described as ''endothelium derived relaxing factor'' (EDRF) is in fact a gas, i.e. nitric oxide (Blantz and Munger, 2002). An isoenzyme of •NO synthase that is stimulated by inflammatory as well as proinflammatory stimuli and is calcium-dependent (several isoforms have been described up until now), i.e. the so-called inducible •NO synthase (iNOS), plays an especially important pathophysiological role (Robbins and Singharajah, 2005; Korhonen et al., 2005). Recent studies have shown that activated PMN also possess a high iNOS activity (Cedergren et al., 2003; Jablonska et al., 2005; Kobayashi et al., 2002; Wheeler et al., 1997; Yu et al., 2004). Once formed, •NO diffuses easily through cell membranes and can serve as a cell-endogenous and intercellular mediator and metabolic modulator both intracellularly, in the extracellular matrix as well as in other perigranulocytic cells.

However, the major pathways by which nitric oxide or polyamine-dependent pathways modulate major cellular functions, PMN dynamic free amino and α-keto acid pools, and important bactericidal functions have not yet been defined. Moreover, our current understanding of the rate of nitric oxide or polyamine utilisation and major pathways of metabolism raises some intriguing questions about potential therapeutic manipulation and whether important PMN antibacterial host defence mechanisms can be altered to the benefit of the individual.

The goals of this study were therefore:

1. To document the effects *of N_ω-nitro-L-arginine-methylester-hydrochloride* [*L-NAME*; an inhibitor of nitric oxide (•NO) synthase], *S-nitroso-N-acetyl-penicillamine* [*SNAP*; an •NO donor] and *α-difluoro-methyl-ornithine* [*DFMO*; an inhibitor of ornithine-decarboxylase], *arginine* or *ornithine* on PMN free intracellular amino- and α-keto acid concentrations as well as on the activities of released myeloperoxidase (MPO) and the formation of superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) (as markers of PMN function and in order to show possible parallels with changes in PMN amino acid concentrations).
2. To explore the effects of *arginine* or *ornithine* (regarding its role in PMN immunonutrition) combined with *L-NAME, SNAP* or *DFMO* on PMN amino- and α-keto acid concentrations or important PMN immune functions in order to establish whether an inhibitor of •NO-synthase, an •NO donor as well as an inhibitor of ornithine-decarboxylase may influence any of the *arginine* or *ornithine*-induced effects.

Materials and methods

The study was approved by the local ethics committee of the University Hospital Giessen and Marburg. Ten men between 25 and 38 years (31 ± 3.9) with an average height of 179.0 cm (range 170–186) and weight of 79.1 kg (range 73–91) were selected: males with metabolic (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 h fasting) taking circadian variation into account.

L-NAME, SNAP or DFMO

The following concentrations were tested in addition to a control for each substance:

1. *L-NAME* (inhibitor of nitric oxide (•NO) synthase): 1 mM [N_ω-nitro-L-arginine-methylester-hydrochloride; Calbiochem, Bad Schwalbach, Germany]
2. *SNAP* (exogenous nitric oxide donor; •NO-release: 5.6 μM/min): 100 μM [S-nitroso-N-acetyl-penicillamine; Sigma, Deisenhofen, Germany]
3. *DFMO* (irreversible inhibitor of ornithine decarboxylase): 1 mM [α-difluoro-methyl-ornithine; Sigma, Deisenhofen, Germany]

PMN were incubated with agents for 10, 60 and 120 min to examine the dynamics of response to these agents. The final concentrations of L-NAME, SNAP or DFMO selected corresponded-consistent with previous work- to those used in our own preliminary studies (not published).

Arginine or ornithine + L-NAME, SNAP and DFMO

The following concentrations were tested in addition to controls (PMN incubation for 120 min:

1. *Arginine* (1 mM):
   - + *L-NAME* (1 mM)
   - + *SNAP* (100 μM)
   - + *DFMO* (1 mM)

2. *Ornithine* (0.5 mM):
   - + *L-NAME* (1 mM)
   - + *SNAP* (100 μM)
   - + *DFMO* (1 mM)

Solutions were prepared and diluted in Hank's balanced salt solution (HBSS; Sigma, Deisenhofen, Germany), and the pH in the test solution was confirmed as 7.4. One millilitre of whole blood was incubated with 25 μl of test solution (final ornithine concentrations were as described above) at 37 °C using a shaking 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 sulphonyl 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 leukocytes (PMN) from whole blood

Precise details of our PMN-separation technique have been described previously (Mühling et al., 1999, 2003). This method is a further development of the methods described by Eggleton et al. (1989) and Krumholz et al.

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

Chromatographic amino and α-keto acid analysis

Amino- and α-keto acids in PMN were quantified using previously described methods which fulfilled the strict criteria required for ultrasensitive, comprehensive amino- and α-keto acid analysis, specially developed and precisely validated in our institute for this purpose (for details see (Mühling et al., 1999, 2003). The coefficients of variations for both the method reproducibility and reproducibilities of the retention times were within normal ranges. PMN amino acid concentrations are given in 10^{-16} moles per PMN-cell, PMN α-keto acid concentrations are given in 10^{-17} moles per PMN-cell.

Superoxide anion (O_2^-), hydrogen peroxide production (H_2O_2) and the activity of released myeloperoxidase (MPO)

These were determined photometrically using modifications of known methods validated in our institute for this purpose (Krumholz et al., 1995, 1993).

Superoxide anion production

This 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. Opsonised 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), opsonised zymosan was resuspended in 10 ml PBS® glucose buffer, portioned and stored at −20 °C. Whole blood was incubated with the test solutions described above. These preparations were then incubated for 10, 60 or 120 min at 37 °C (shaking water bath). The PMN were then isolated using a modified PMN separation technique as described above. After stepwise (15 min and 5 min) centrifugation procedures (350 × *g*, 20 °C), as well as careful lysis of the few remaining erythrocytes that contaminated 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 test solutions 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 samples were prepared, incubated and measured in the same way.

Hydrogen peroxide production

This was also determined photometrically using a method based on horseradish peroxidase catalysed 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 opsonised 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 test solution for 10, 60 or 120 min at 37 °C, PMN were isolated as described above. Isolated PMN were stimulated by opsonised zymosan. The final preparation consisted of 500 µl zymosan, 125 µl pooled 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 test solutions (15 min, 37 °C). After adding 25 µl 1N sodium hydroxide solution (Merck, Darmstadt, Germany), the extinction was measured photometrically at 623 nm. All control samples were prepared, incubated and measured in the same way.

Activity of released myeloperoxidase

1 mM 2,2′-azino-di-(3-ethyl-benzthiazoline) sulphonic acid (ABTS, Sigma, Deisenhofen, Germany) was dissolved in 0.1 M citrate buffer (Behring, Marburg, Germany; pH 7.4). The preparation of whole blood was incubated with test solutions either for 10, 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 test solutions for 5 min (37 °C). After addition of 100 µl opsonised zymosan and supplementation of agents in order to keep their 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 co-operation with colleagues from the Department of Medical Statistics, Justus Liebig University Giessen.

All tests were performed in duplicate. Thus our PMN amino acid results represent 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 Friedmann test was performed. Probability levels of $p \leq 0.05$ versus control were considered as significant. The data are given as arithmetic means ± standard deviations (mean ± SD).

Results

The free intracellular amino and α-keto acid concentrations, superoxide anion and hydrogen peroxide generation rates and the activities of released myeloperoxidase obtained in the control cells were within normal physiological ranges (see Krumholz et al., 1993, 1995).

L-NAME

1. Effects of L-NAME on the free amino acid or α-keto acid pools in PMN

Concentrations of free intracellular amino- as well as α-keto acids were unaffected by 1 mM L-NAME (PMN incubation for 10, 60 or 120 min; data not shown).

2. Effects of L-NAME on oxidative response and myeloperoxidase activity

L-NAME (1 mM) significantly increased both superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) formation (PMN incubation for ≥30 min), while myeloperoxidase activity remained unaltered (Table 4).

SNAP

1. Effects of SNAP on the free amino acid or α-keto acid pools in PMN

Following 100 μM SNAP (PMN incubation for ≥60 min), significant decreases in PMN asparagine, glutamine, aspartate, glutamate, ornithine, arginine, citrulline (Table 1) as well as in PMN alanine, hypotaurine, serine, glycine and threonine (Table 2), were observed. Moreover, 100 μM SNAP (PMN incubation for ≥60 min) significantly increased PMN isoleucine, leucine, and valine (Table 2). PMN free lysine, methionine, taurine, α-aminobutyrate, tyrosine, tryptophan, phenylalanine and histidine profiles remained unaffected. Moreover, following a short duration of SNAP application (10 min; SNAP 100 μM), free intracellular α-keto acid profiles remained unaffected. In the presence of 120 min whole blood incubation with SNAP (100 μM) significant decreases in PMN α-ketoglutarate (KG), pyruvate (PYR), α-ketobutyrate (KB), α-ketoisovaleriate (KIV), α-ketoisocaproate (KIC), p-hydroxy-phenylpyruvate (PhePYR) and α-keto-β-methylvaleriate occurred (KMV; Table 3).

Table 1. Effects of *S-nitroso-N-acetyl-penicillamine* (SNAP; 100 μM) and *α-difluoro-methyl-ornithine* (DFMO; 1 mM) incubated with whole blood for 10, 60 and 120 min on free intracellular *acidic amino acid*, *acidic amide* and *basic amino acid* concentrations in PMN (10^{-16} moles per PMN-cell; mean ± SD; n = 10)

Acidic amino acids, acidic amides, basic amino acids			
	Control	SNAP	DFMO
10 min			
Asn	0.37 ± 0.06	0.35 ± 0.06	0.39 ± 0.07
Gln	2.80 ± 0.58	2.66 ± 0.50	2.84 ± 0.57
Asp	2.83 ± 0.45	2.53 ± 0.48	2.78 ± 0.46
Glu	5.90 ± 0.86	6.04 ± 0.87	5.75 ± 0.85
Orn	0.45 ± 0.07	0.43 ± 0.08	0.44 ± 0.08
Arg	0.32 ± 0.06	0.29 ± 0.06	0.31 ± 0.06
Cit	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
60 min			
Asn	0.38 ± 0.06	0.33 ± 0.06	0.35 ± 0.05
Gln	2.88 ± 0.60	2.25 ± 0.47*	2.41 ± 0.47
Asp	2.94 ± 0.48	2.36 ± 0.42*	2.58 ± 0.17
Glu	6.01 ± 0.88	4.57 ± 0.80*	5.33 ± 0.85
Orn	0.46 ± 0.08	0.42 ± 0.08	0.57 ± 0.10*,#
Arg	0.33 ± 0.07	0.30 ± 0.06	0.36 ± 0.07
Cit	0.12 ± 0.03	0.11 ± 0.02	0.13 ± 0.02
120 min			
Asn	0.36 ± 0.05	0.28 ± 0.05*,#	0.31 ± 0.05#
Gln	2.70 ± 0.56	1.88 ± 0.40*,#	2.15 ± 0.45*,#
Asp	2.72 ± 0.43	1.96 ± 0.46*,#	2.27 ± 0.40*,#
Glu	5.79 ± 0.85	4.15 ± 0.78*,#	4.78 ± 0.84*,#
Orn	0.47 ± 0.09	0.38 ± 0.07*,#	0.73 ± 0.13*,#
Arg	0.32 ± 0.06	0.26 ± 0.05*,#	0.39 ± 0.07*,#
Cit	0.11 ± 0.02	0.09 ± 0.02*,#	0.15 ± 0.03*,#

* p ≤ 0.05 versus control values; # p ≤ 0.05 versus 10 min

Table 2. Effects of *S-nitroso-N-acetyl-penicillamine* (SNAP; 100 μM) and *α-difluoro-methyl-ornithine* (DFMO; 1 mM) incubated with whole blood for 10, 60 and 120 min on free intracellular *branched chain amino acid* (*BCAA*), *hypotaurine* and *neutral amino acid* concentrations in PMN (10^{-16} moles per PMN-cell; mean ± SD; n = 10)

BCAA, hypotaurine, neutral amino acids			
	Control	SNAP	DFMO
10 min			
Ile	0.40 ± 0.07	0.39 ± 0.07	0.39 ± 0.07
Leu	0.69 ± 0.16	0.70 ± 0.18	0.65 ± 0.15
Val	0.58 ± 0.12	0.52 ± 0.12	0.54 ± 0.13
H-Tau	1.54 ± 0.34	1.43 ± 0.30	1.69 ± 0.26
Ser	2.29 ± 0.45	2.18 ± 0.43	2.30 ± 0.48
Gly	2.39 ± 0.50	2.30 ± 0.49	2.39 ± 0.51
Thr	0.73 ± 0.12	0.67 ± 0.11	0.71 ± 0.11
Ala	1.80 ± 0.36	1.69 ± 0.34	1.90 ± 0.40
60 min			
Ile	0.39 ± 0.07	0.47 ± 0.08*	0.44 ± 0.08
Leu	0.70 ± 0.15	0.84 ± 0.17	0.80 ± 0.16
Val	0.55 ± 0.11	0.64 ± 0.13	0.64 ± 0.14
H-Tau	1.30 ± 0.32	1.11 ± 0.29	1.22 ± 0.29
Ser	2.19 ± 0.43	2.06 ± 0.43	2.01 ± 0.40
Gly	2.30 ± 0.49	1.97 ± 0.45	2.08 ± 0.45
Thr	0.69 ± 0.11	0.63 ± 0.10	0.64 ± 0.11
Ala	1.79 ± 0.32	1.46 ± 0.31	1.70 ± 0.30
120 min			
Ile	0.38 ± 0.07	0.54 ± 0.10*,#	0.48 ± 0.09*,#
Leu	0.67 ± 0.13	0.98 ± 0.18*,#	0.84 ± 0.17*,#
Val	0.59 ± 0.11	0.82 ± 0.15*,#	0.73 ± 0.13*,#
H-Tau	1.39 ± 0.21	1.02 ± 0.24*,#	1.17 ± 0.23*
Glu	5.79 ± 0.85	4.15 ± 0.78*,#	4.15 ± 0.78*,#
Ser	2.08 ± 0.41	1.49 ± 0.31*,#	1.69 ± 0.36*,#
Gly	2.32 ± 0.47	1.56 ± 0.35*,#	1.79 ± 0.41*,#
Thr	0.66 ± 0.10	0.50 ± 0.09*,#	0.56 ± 0.10*,#
Ala	1.98 ± 0.32	1.40 ± 0.35*,#	1.65 ± 0.31*,#

* p ≤ 0.05 versus control values; # p ≤ 0.05 versus 10 min

2. Effects of SNAP on oxidative response and myeloperoxidase activity

In the presence of SNAP (100 μM; PMN incubation for ≥60 min), PMN superoxide anion and hydrogen peroxide production and MPO activity decreased significantly (Table 4).

DFMO

1. Effects of DFMO on the free amino acid or α-keto acid pools in PMN

Following 1 mM DFMO (PMN incubation for 120 min), significant decreases in PMN asparagine, glutamine,

Table 3. Effects of *S-nitroso-N-acetyl-penicillamine* (SNAP; 100 μM) and *α-difluoro-methyl-ornithine* (DFMO; 1 mM) incubated with whole blood for 10 and 120 min on free intracellular *α-keto acid [α-ketoglutarate (α-KG), pyruvate (PYR), α-ketobutyrate (α-KB), α-ketoisovalerianate (α-KIV), α-ketoisocapronate (α-KIC), p-hydroxy-phenylpyruvate (PhePYR) and α-keto-β-methylvalerianate (α-KMV)]* concentrations in PMN (10^{-17} moles per PMN-cell; mean ± SD; n = 10)

α-Keto acids			
	Control	SNAP	DFMO
10 min			
α-KG	1.37 ± 0.27	1.30 ± 0.26	1.16 ± 0.24
PYR	5.96 ± 1.19	6.23 ± 1.39	5.75 ± 1.18
α-KB	4.20 ± 0.89	4.07 ± 0.94	3.87 ± 0.84
α-KIV	2.20 ± 0.49	2.06 ± 0.51	2.03 ± 0.45
α-KIC	0.38 ± 0.08	0.34 ± 0.07	0.35 ± 0.07
PhePYR	0.31 ± 0.07	0.30 ± 0.06	0.28 ± 0.06
α-KMV	0.80 ± 0.16	0.78 ± 0.16	0.70 ± 0.15
120 min			
α-KG	1.19 ± 0.24	0.71 ± 0.13*,#	0.88 ± 0.20*,#
PYR	5.76 ± 1.03	3.87 ± 0.72*,#	4.79 ± 1.01*,#
α-KB	4.39 ± 0.86	3.18 ± 0.63*,#	3.60 ± 0.84*
α-KIV	2.31 ± 0.38	1.74 ± 0.41*	1.95 ± 0.43*
α-KIC	0.36 ± 0.07	0.26 ± 0.06*,#	0.30 ± 0.06*
PhePYR	0.32 ± 0.07	0.25 ± 0.05*	0.28 ± 0.06
α-KMV	0.75 ± 0.13	0.55 ± 0.12*,#	0.63 ± 0.14*

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

aspartate, glutamate, ornithine, arginine, citrulline (Table 1) as well as in PMN alanine, hypotaurine, serine, glycine and threonine (Table 2), were observed. Moreover, 1 mM DFMO (PMN incubation for 120 min) significantly increased PMN isoleucine, leucine, and valine (Table 2). PMN free lysine, methionine, taurine, α-aminobutyrate, tyrosine, tryptophan, phenylalanine and histidine profiles remained unaffected. Moreover, following a short duration of SNAP application (10 min; DFMO 1 mM), free intracellular α-keto acid profiles remained unaffected. In the presence of 120 min whole blood incubation with SNAP (100 μM) significant decreases in PMN KG, PYR, KB, KIV, KIC and KMV occurred (Table 3).Only PhePYR profiles remained unaffected.

2. Effects of DFMO on oxidative response and myeloperoxidase activity

In the presence of SNAP (100 μM; PMN incubation for ≥60 min), PMN superoxide anion and hydrogen peroxide generation as well as released MPO activity decreased significantly (Table 4).

Arginine/ornithine + L-NAME, SNAP or DFMO

1. Effects of arginine/ornithine + L-NAME, SNAP or DFMO on free amino acid pools in PMN

Arginine (1 mM) or ornithine (0.5 mM) led to a significant increase in PMN asparagine (only ornithine), glutamine, aspartate, glutamate, ornithine, arginine, citrulline as well as in PMN alanine (Figs. 1 to 4). L-NAME did not induce

Table 4. Effects of *N_ω-nitro-L-arginine-methylester-hydrochloride* (L-NAME; 1 mM), *S-nitroso-N-acetyl-penicillamine* (SNAP; 100μM) and *α-difluoro-methyl-ornithine* (DFMO; 1 mM) incubated with whole blood for 10, 60 and 120 min on PMN superoxide anion (O_2^-) generation [fMol O_2^-/(PMN × min)], hydrogen peroxide (H_2O_2) production [fMol H_2O_2/(PMN × min)] and on PMN myeloperoxidase (*MPO*) activity ([Units/l supernatant]; mean ± SD; n = 10)

PMN immune functions				
	Control	L-NAME	SNAP	DFMO
10 min				
O_2^-	3.536 ± 0.551	4.147 ± 0.703	3.275 ± 0.618	3.498 ± 0.545
H_2O_2	1.324 ± 0.225	1.428 ± 0.294	1.302 ± 0.236	1.260 ± 0.287
MPO	0.685 ± 0.175	0.609 ± 0.168	0.637 ± 0.182	0.648 ± 0.169
60 min				
O_2^-	3.461 ± 0.538	5.077 ± 0.863*,#	2.537 ± 0.598*,#	2.875 ± 0.523*
H_2O_2	1.239 ± 0.219	1.732 ± 0.401*	0.854 ± 0.182*,#	1.017 ± 0.219*
MPO	0.646 ± 0.161	0.586 ± 0.159	0.469 ± 0.144*	0.506 ± 0.145
120 min				
O_2^-	3.317 ± 0.528	5.794 ± 1.151*,#	2.106 ± 0.483*,#	2.593 ± 0.508*,#
H_2O_2	1.154 ± 0.195	2.123 ± 0.522*,#	0.697 ± 0.171*,#	0.814 ± 0.184*,#
MPO	0.605 ± 0.153	0.501 ± 0.140	0.315 ± 0.099*,#	0.371 ± 0.108*,#

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

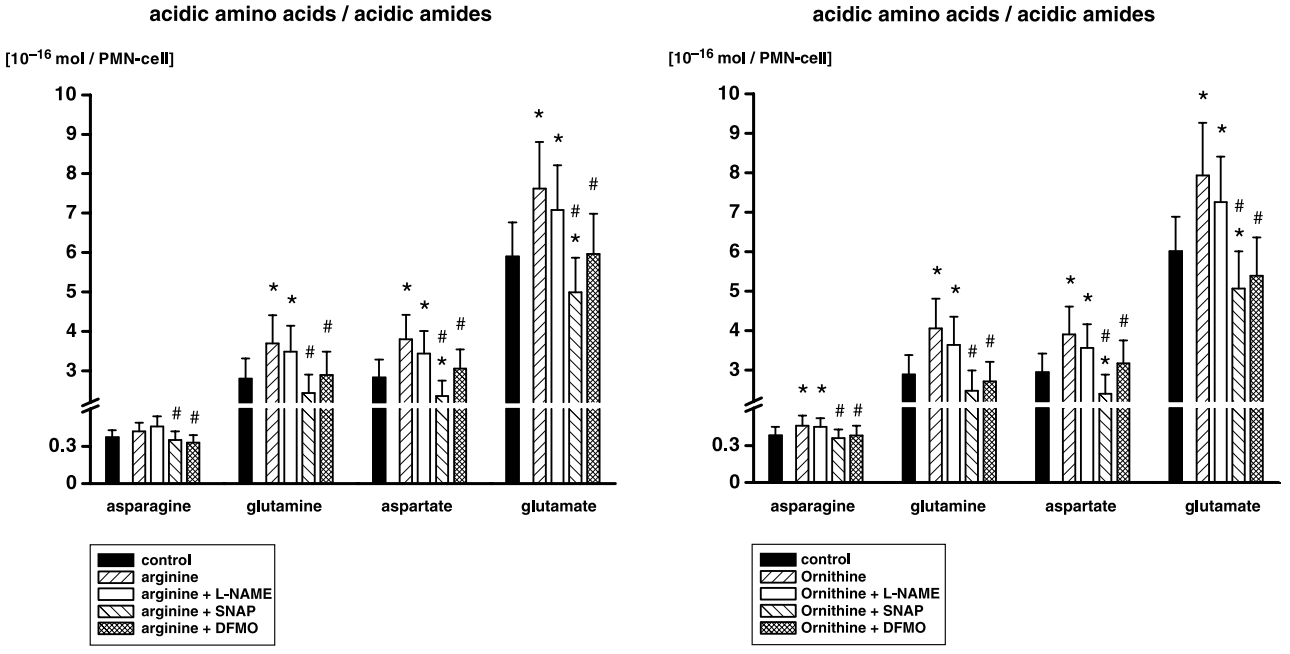

Fig. 1. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with N_{ω}-*nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 µM)] or *α-difluoro-methyl-ornithine* [DFMO, (1 mM)] on free intracellular *acidic amino acid* and *acidic amide* concentrations in PMN (10^{-16} moles per PMN-cell; mean ± SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

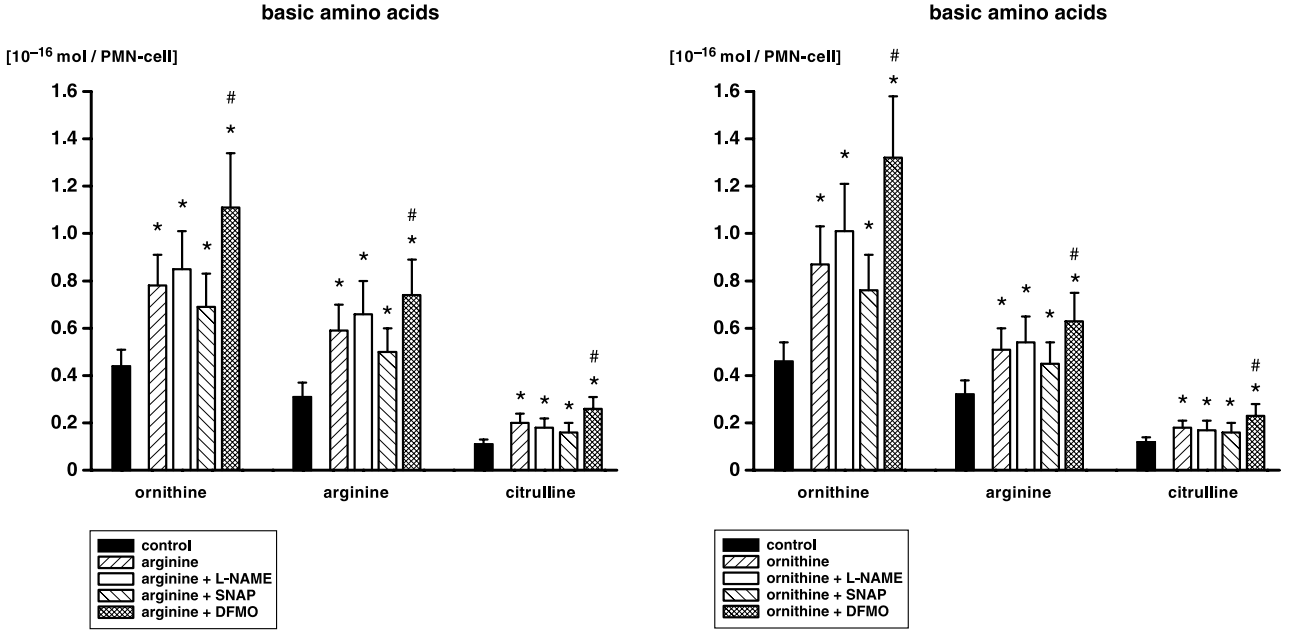

Fig. 2. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with N_{ω}-*nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 µM)] or *α-difluoro-methyl-ornithine* [DFMO, (1 mM)] on free intracellular *basic amino acid* concentrations in PMN (10^{-16} moles per PMN-cell; mean ± SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

any effects exceeding those of arginine or ornithine single incubation (Figs. 1 to 4). The combined administration of SNAP (100 µM; Figs. 1 to 4) and arginine or ornithine led to a cancellation of the arginine or ornithine effect with asparagine (only ornithine), glutamine and alanine. SNAP did not have any influence, however, on the

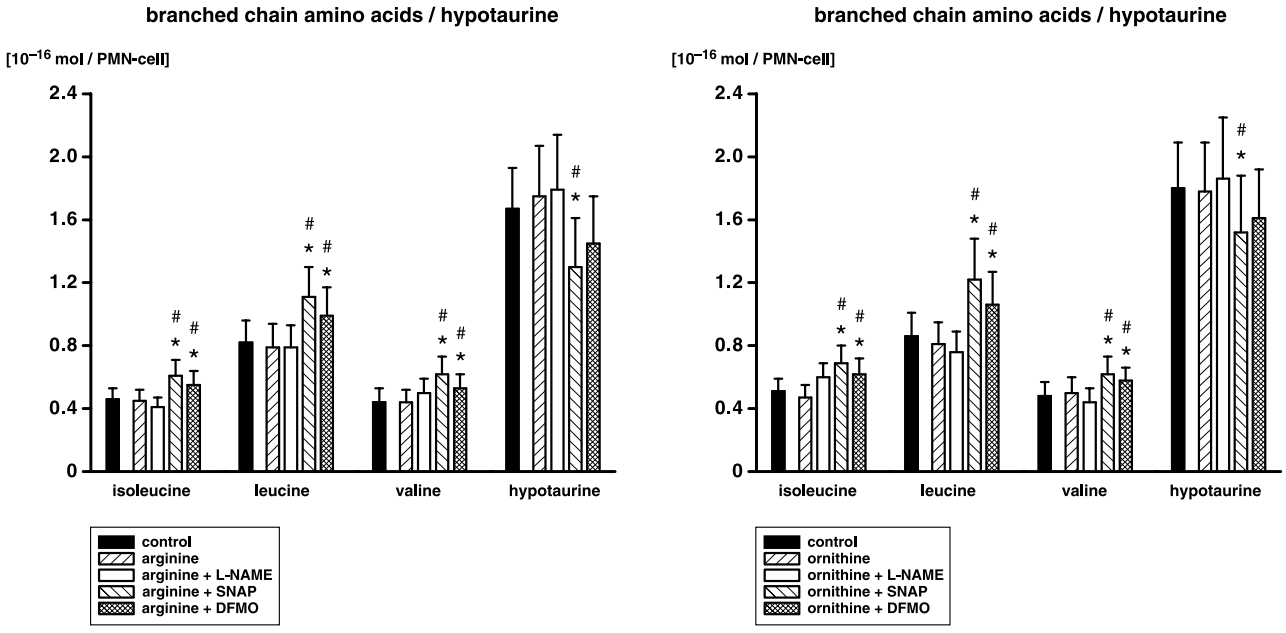

Fig. 3. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with N_{ω}-*nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 μM)] or α-*difluoro-methyl-ornithine* [DFMO, (1 mM)] on free intracellular *branched chain amino acid* (*BCAA*) and *hypotaurine* concentrations in PMN (10^{-16} moles per PMN-cell; mean ± SD; SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

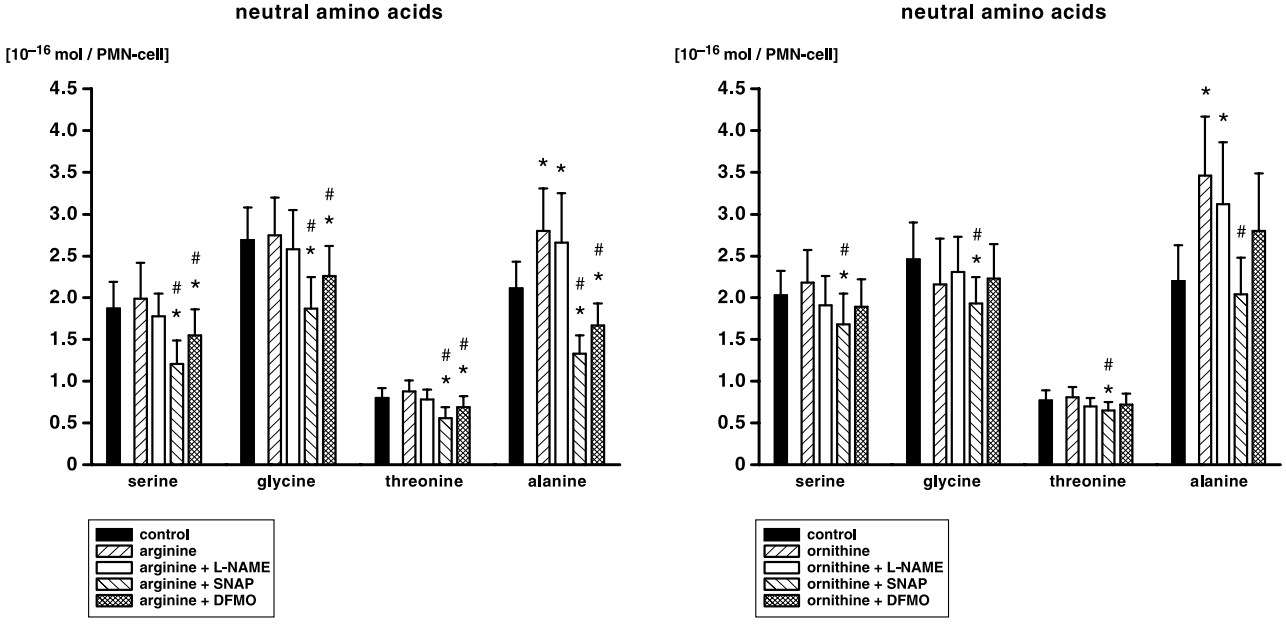

Fig. 4. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with N_{ω}-*nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 μM)] or α-*difluoro-methyl-ornithine* [DFMO, (1 mM)] on free intracellular *neutral amino acid* concentrations in PMN (10^{-16} moles per PMN-cell; mean ± SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

arginine or ornithine-induced alterations in the basic amino acids. Additional significant reductions of the intracellular amino acid concentrations to below control levels (Arg/Orn + SNAP) were found with aspartate, glutamate, hypotaurine, serine, glycine and threonine. Moreover, SNAP combined with arginine or ornithine significantly increased

PMN isoleucine, leucine and valine. DFMO (1 mM; Figs. 1–4) combined with arginine or ornithine led to a cancellation of the arginine or ornithine single effects with asparagine (only ornithine), glutamine, aspartate, glutamate and alanine. In addition, the parallel incubation of DFMO with arginine or ornithine led to further increases in intracellular arginine, ornithine and citrulline levels. Consistent with the findings from a single application of DFMO, combined administration of DFMO and arginine or ornithine led to significant increases in PMN branched chain amino acids as well as decreases in intracellular neutral amino acid levels. Compared with SNAP, however, the effects were weaker. Moreover, following arginine or ornithine combined with L-NAME, SNAP or DFMO PMN free lysine, methionine, taurine, α-aminobutyrate, tyrosine, tryptophan, phenylalanine and histidine profiles remained unaffected.

2. Effects of arginine/ornithine + L-NAME, SNAP or DFMO on free α-keto acid pools in PMN

Arginine (1 mM) or ornithine (0.5 mM) led to a significant increase in intracellular KG and PYR concentrations (Fig. 5). The combination with L-NAME (1 mM), however, did not produce an effect exceeding that of arginine or ornithine single administration alone. On the other hand, additional administration of SNAP (100 μM) to arginine or ornithine led to a cancellation of the arginine or ornithine effects with an almost complete reduction of intracellular concentrations of all α-keto acids tested here (Figs. 5 and 6). A similar, but overall weaker effect was seen with arginine + DFMO (1 mM; Figs. 5 and 6). Whereas in combination with arginine, a cancellation of the arginine effect with KG and PYR as well as a reduction of other important α-keto acids could be shown, DFMO in combination with ornithine only led to a cancellation of ornithine-induced KG and PYR increases, with all other examined α-keto acids remaining unchanged (Fig. 5).

3. Effects of arginine or ornithine + L-NAME, SNAP or DFMO on oxidative response and myeloperoxidase activity

In the presence of arginine (1 mM) or ornithine (0.5 mM), superoxide anion generation decreased significantly. Moreover, both amino acids caused significant increases in hydrogen peroxide formation and MPO activity (Fig. 7). The combination of arginine or ornithine with L-NAME (1 mM; Fig. 7) led here to a complete cancellation of the ornithine or arginine-induced reduction in superoxide anion generation with significant increases in this parameter

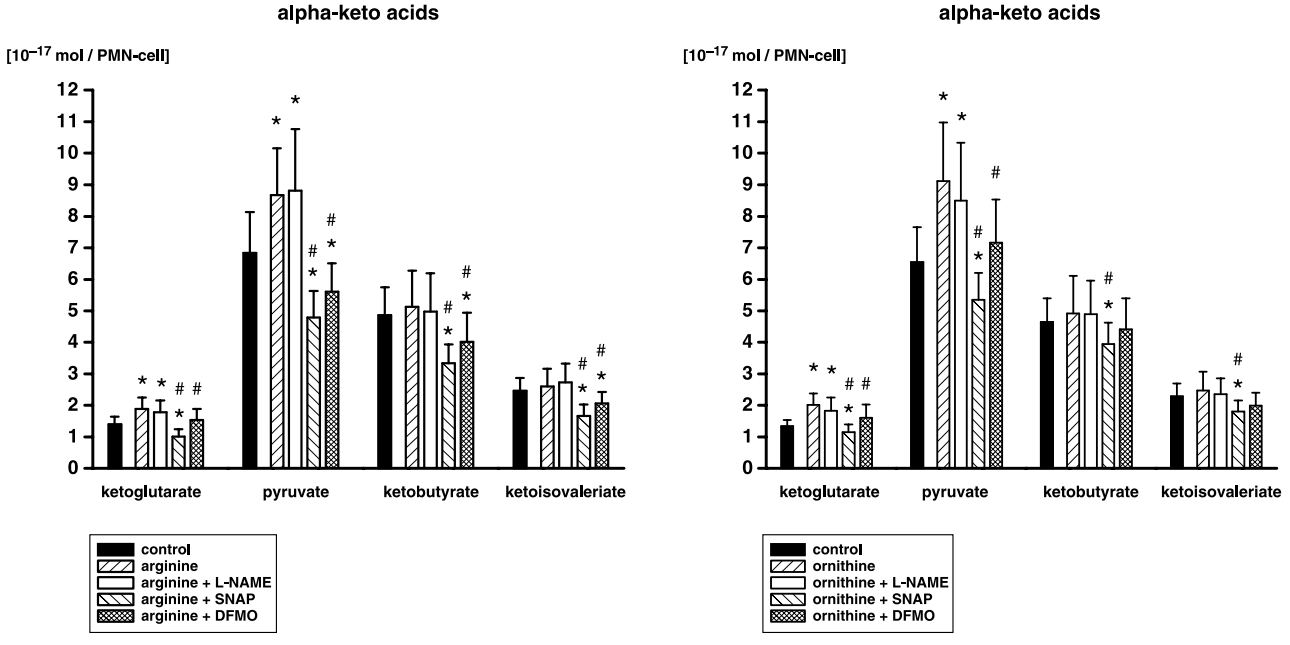

Fig. 5. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with *N_ω-nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 μM)] or *α-difluoro-methyl-ornithine* [DFMO, (1 mM)] on free intracellular *α-ketoglutarate*, *pyruvate*, *α-ketobutyrate* and *α-ketoisovalerianate* concentrations in PMN (10^{-17} moles per PMN-cell; mean ± SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

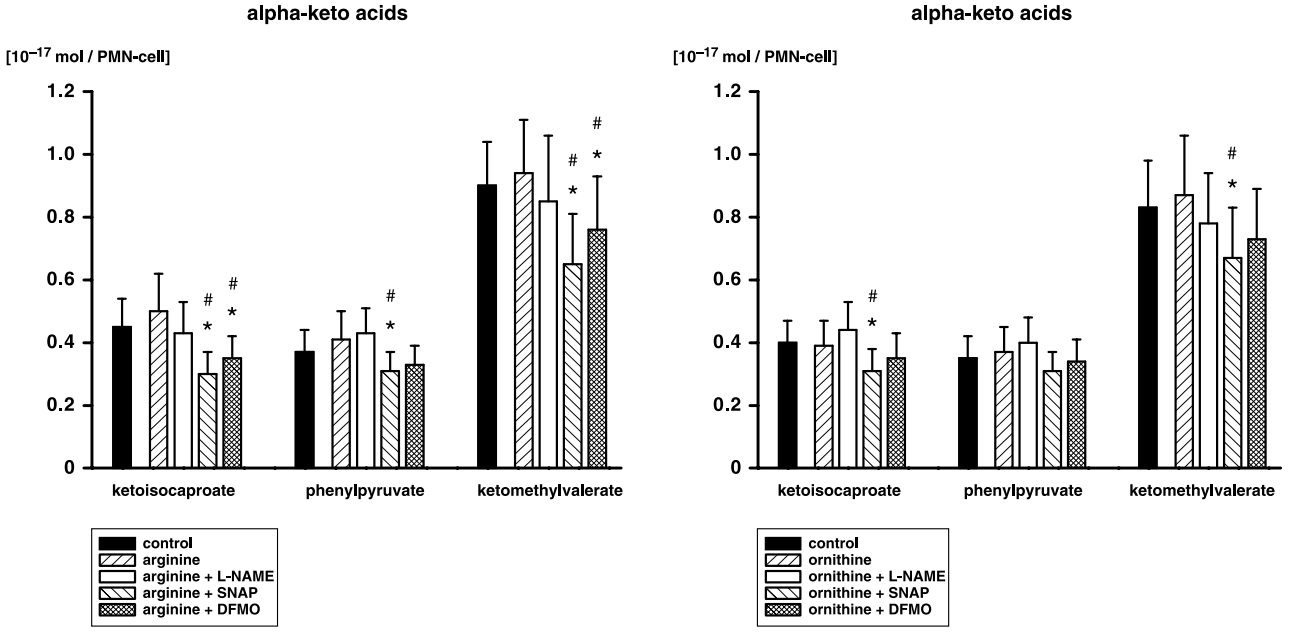

Fig. 6. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with N_{ω}*-nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 μM)] or α*-difluoro-methyl-ornithine* [DFMO, (1 mM)] on free intracellular α*-ketoisocapronate*, *p-hydroxy-phenylpyruvate* and α*-keto-β-methylvalerianate* concentrations in PMN (10^{-17} moles per PMN-cell; mean ± SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

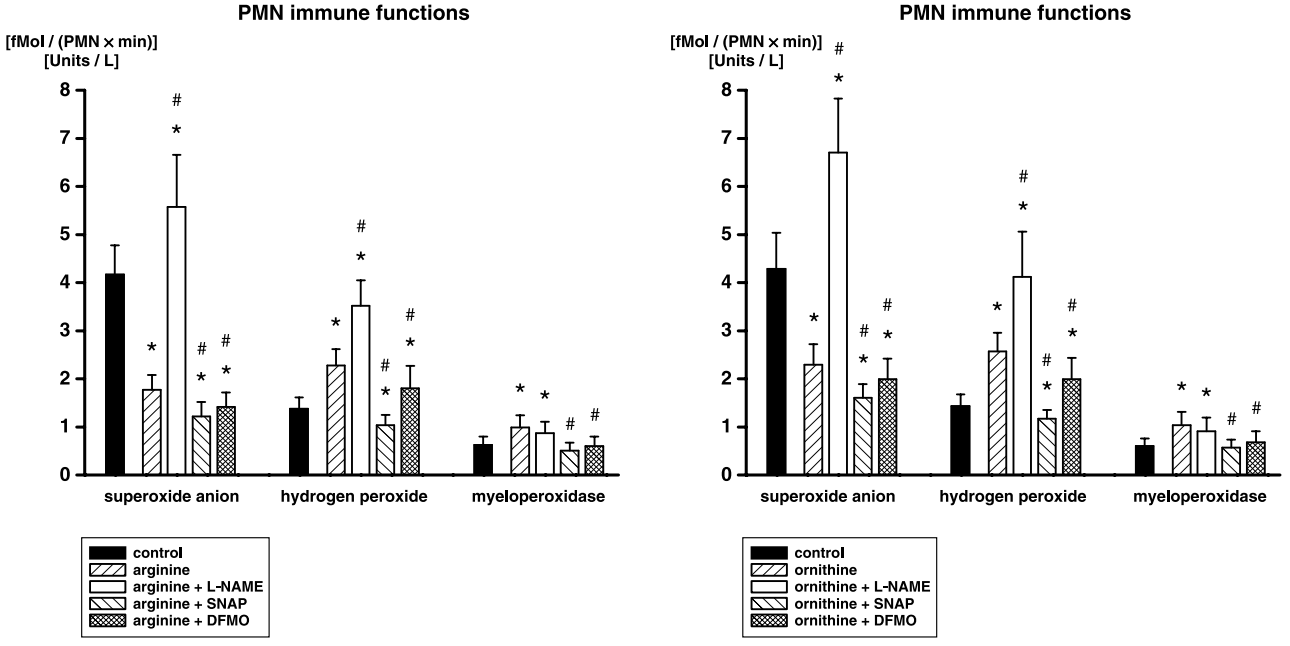

Fig. 7. Effects of *arginine* (1 mM) or *ornithine* (0.5 mM) incubated with whole blood for 120 min combined either with N_{ω}*-nitro-L-arginine-methylester-hydrochloride* [L-NAME (1 mM)], *S-nitroso-N-acetyl-penicillamine* [SNAP (100 μM)] or α*-difluoro-methyl-ornithine* [DFMO, (1 mM)] on PMN superoxide anion generation [fMol O_2^-/(PMN × min)], hydrogen peroxide production [fMol H_2O_2/(PMN × min)] and on PMN myeloperoxidase activity ([Units/l supernatant]; mean ± SD; n = 10). * $p \leq 0.05$ versus control values; # $p \leq 0.05$ versus arginine or ornithine

above control levels. In addition, when combining arginine or ornithine with L-NAME, additive increases were observed in hydrogen peroxide formation. No differences compared to single arginine or ornithine incubation were found, however, regarding the MPO activity. SNAP (100 μM; Fig. 7) in combination with arginine or ornithine

led to additive effects regarding superoxide anion generation as well as a cancellation of arginine or ornithine effects with regard to hydrogen peroxide formation (under control levels) and MPO activity. These SNAP typical findings were also found when arginine or ornithine was incubated with DFMO (1 mM). However, compared with SNAP, the effects of DFMO on the immune parameters examined here were clearly lower (Fig. 7).

Discussion

As the findings from the present study show, the intragranulocytic arginine and ornithine concentrations were 0.3–0.4 and 0.4–0.5 $\times 10^{-16}$ moles per PMN cell, respectively. For arginine almost plasmaisotonic conditions (arginine: ≈66–88 μM/l PMN cell volume) prevail, while for ornithine (≈88–110 μM/l PMN cell volume) there is a high intra versus extracellular concentration ratio (2–3:1; Mühling et al., 1999). This is worthy of note because arginine in human plasma has almost twice the concentration that ornithine has [arginine: ≈80 μMol/l; ornithine ≈40 μMol/l (Bender, 1985)]. This may on the one hand be due to a selective granulocytic accumulation of ornithine, but it could also be due to a high intracellular synthetic rate for this amino acid. Indeed, various authors found no relevant differences in the transmembrane transport of both amino acids, also in PMN cells, so that an increased uptake of ornithine from the extracellular matrix would not appear to be primarily responsible for the observed intragranulocytic ornithine accumulation (Bansal and Ochoa, 2003; Tong and Barbul, 2004a; Evoy et al., 1998; Moinard et al., 1999). Apart from the transmembranous uptake of both amino acids from the extracellular spaces, intracellular arginine and ornithine synthesis also occurs in the PMN cells (Chen and Mehta, 1996; Evoy et al., 1998; Moffat et al., 1996; Schapira et al., 1998). The turntable for intragranulocytic arginine and ornithine metabolism, as earlier studies showed, appears to be the intragranulocytic urea cycle whose cell-specific enzyme activity can be primarily indicted as a cause underlying the intracellular ornithine accumulation observed. Arginase in particular shows a high (mean: 1644 ± 423 mU/mg protein) intragranulocytic activity constitutively (Evoy et al., 1998; Munder et al., 2005; Waddington et al., 1998). Moreover, by analysis of purified granulocytes from patients with arginase I-deficiency, it was proven that human granulocytes express the hepatic isoform of arginase (arginase I) only and not the extrahepatic arginase II (Munder et al., 2005). Amazingly, the activity of this enzyme differs greatly between different cell types, a fact which seems far from arbitrary in nature (Yu et al., 2003). As different studies have shown, a tight (inverse) correlation certainly exists between intracellular arginase activity and cellular arginine and particularly ornithine content (Kriegbaum et al., 1987; Reyero and Dorner, 1975). Thus in kidney as well as muscle cells there is as an almost 10-fold higher intracellular arginine concentration and a 50–100 fold reduced arginase activity compared to PMN or liver cells (Fuchs et al., 1994a, b). Liver cells show a particularly tight inverse correlation between arginase activity and intracellular arginine content (Laszlo et al., 1991). The reasons for this remain largely unclarified. One metabolic correlate might be related for example to the appropriation of ornithine for polyamine synthesis (Igarashi and Kashiwagi, 2000; Satriano, 2004). Polyamines are essential metabolic components for almost all cell types with high cell division rates and metabolic activities; for this reason they are particularly prevalent in leukocytes and PMN cells (Guarnieri et al., 1987; Moinard et al., 2002a, b). In order to maintain intragranulocytic pools, polyamines are both taken up from the extracellular matrix and newly synthesised by the enzymatic decarboxylation of ornithine (via ornithine decarboxylase; Igarashi and Kashiwagi, 2000). However, the largest proportion of cellular polyamines in PMN is newly synthesised since the membranous transport mechanism for polyamines has only a low capacity (Grillo and Colombatto, 1994). The putrescine (a diamine) arising from ornithine decarboxylase is itself a precursor for the metabolically active polyamines spermidine and spermine, enzymatically converted by the spermidine synthase reaction that is also detectable in leukocytes (Das, 1994). Their biological roles, especially regarding their specific metabolic functions in human leukocytes, remain virtually unexplored. It is assumed that polyamines have specific functions in cellular growth, proliferation and apoptotic processes, e.g. the stabilisation of cellular DNA and RNA through the binding of spermine and spermidine at their acid phosphate groups, but possibly also the modulation of cellular protein biosynthesis (Igarashi and Kashiwagi, 2000; Bansal and Ochoa, 2003). According to the findings of various authors, polyamines especially in leukocytes appear also to fulfil immunofunctional and immunomodulatory roles (Guarnieri et al., 1987; Lovaas, 1995; Walters et al., 1998, 1995a, b). An impairment or even interruption of cellular polyamine synthesis, as results here show, cannot insignificantly influence other basic metabolic processes, such as the granulocytic immune response. Studies in which a reduction of intracellular polyamine synthesis was brought about by inhibition of the key

enzyme responsible for synthesis, e.g. by using an ornithine decarboxylase inhibitor [α-difluoro-methyl-ornithine (DFMO)], confirmed this hypothesis (Tripathi et al., 2002; Walters et al., 1998, 1995a, b). Such a pharmacologically-induced impairment of intracellular polyamine synthesis leads to a modulation of the leucocyte immune response with changes in the secretion of reactive oxygen metabolites, granulocytic vesicles and granula as well as pro-inflammatory mediators, and also to modifications in cell- and tissue-specific anti-inflammatory protective mechanisms (Moinard et al., 2005; Grillo and Colombatto, 2004; Grimble and Grimble, 1998). According to the results presented here a blockade of granulocyte ornithine decarboxylation by DFMO leads to important modifications in intragranulocytic amino- and α-keto acid levels and, consistently with earlier studies, a reduction in superoxide anion and hydrogen peroxide generation as well as a decrease in extragranulocytically secreted myeloperoxidase activity (Guarnieri et al., 1987; Kafy et al., 1986; Walters et al., 1995a, b, 1998). Apart from the inhibition of intracellular ornithine metabolisation, a far-reaching modification of other cellular metabolic processes (e.g. glutamate metabolisation) with a direct influencing on the free intragranulocytic amino acid pools must also be postulated as a cause for the observed changes in amino- and α-keto acids. In addition, a lasting inhibition of granulocytic polyamine synthesis appears to influence the further metabolisation of intracellular amino acids to their metabolically active α-keto acids.

Moreover, according to the results presented here as well as former findings we suggest that, apart from cellular polyamine synthesis, arginine and ornithine may also have other important functions as cellular metabolic precursors for important anabolic and catabolic processes. The metabolisation of arginine and ornithine to aspartate (i.e. via fumarate and oxalacetate as well as via pyruvate), but also to glutamate and α-ketoglutarate (i.e. via glutamate-γ-semialdehyde or derivatised via the ornithine-α-keto acid transaminase reaction) in different cell types represent important reactions in substrate and amino acid metabolism. This option results from the tight associations of the urea cycle with other intracellular (amino acid) metabolic pathways (Mühling et al., 2001a). The efficiency of the urea cycle (i.e. in leukocytes such as PMN) is significantly improved by the close functional coordination with glutamate metabolisation, as brought about by transaminase, glutaminase or glutamine synthetase reactions (Vlaho and Sieberth, 1981; Wolfe and Gatfield, 1975). This is of great importance for cellular metabolism, because glutamate nevertheless (alongside aspartate and alanine) represents an important turntable for intracellular amino nitrogen metabolism. Glutamine and alanine are not just formed as a result of anabolism, but also because ammonia, generated from arginine or ornithine that is no longer required intracellularly, must be fixed in a form that is not toxic for the cell. In the same way close metabolic relationships exist with the citric acid cycle from the urea cycle metabolite argininosuccinate. According to the results presented here, this is the first study in which evidence for such metabolic associations have also been found for PMN. As expected, arginine (as well as ornithine) supplementation induced direct dose-as well as incubation time-dependent increases in intragranulocytic arginine or ornithine content. Parallel increases in granulocyte citrulline, asparagine, glutamine, glutamate and alanine levels and the α-keto acids pyruvate and α-ketoglutarate metabolised from them were also found. Citrulline appears to be formed, as with other leukocytes, from the enzymatically-mediated and carbamyl phosphate-dependent conversion of ornithine (via ornithine transcarbamylase; Korman et al., 2004). A second possibility for citrulline formation results from the enzymatic transformation of arginine with formation of citrulline and •NO by nitric oxide (•NO) synthase (Tong et al., 2004). Regarding the various known isoforms of •NO synthase, they can be distinguished in essence by the nature of their expression and their enzyme activity. Constitutively occurring •NO synthases, e.g. the calcium-dependent endothelial (type III) and neural (type I) isoforms, produce continuously low quantities (nanomolar concentrations) of citrulline and •NO, while the so-called inducible form, induced by inflammatory processes and mediators and which represents the major form in leukocytes, produces even micromolar amounts of both metabolites (Cedergren et al., 2003; Jablonska et al., 2005). While the presence of a baseline expressed isoform in PMN can only be postulated up until now, when activation occurs, e.g. by cytokines, (pro)inflammatory mediators or lipopolysaccharides, massively increased intragranulocytic enzyme activities of the inducible •NO synthase isoform become evident (Kurizaki et al., 2004; Wallerath et al., 1997; Wheeler et al., 1997). The •NO formed in this way is lipophilic, can diffuse unhindered through cell membranes, and appears to be an important intra- and extracellular metabolite of major pathophysiological importance for many metabolic regulatory processes and immunological functions (Sethi and Dikshit, 2000). The possibility to convert intracellularly accumulated arginine and ornithine to •NO, and also to influence the above described anabolic effects of both amino acids regarding intragranulocytic

substrate and polyamine content, may have a direct influence on granulocytic immune function. According to findings presented here, addition of arginine or ornithine can lead to a parallel reduction of extracellularly secreted superoxide anions, to an increase in hydrogen peroxide generation and an increase in extragranulocytic myeloperoxidase activity. These interesting, but apparently contradictory effects that confirm earlier findings require a more detailed analysis (Reynolds et al., 1988; Salvemini et al., 2003; Seth et al., 1994; Wiedermann et al., 1993). Consistent with our own results, other investigations have shown that both amino acids (administered e.g. in the diet) induce fundamental anabolic effects with significant increases in immunofunctional metabolic reactions, cell functions (e.g. phagocytosis) as well as granulocyte enzyme activities (e.g. myeloperoxidase) (Evoy et al., 1998; Grimble, 2001; Moinard et al., 1999; Suchner et al., 2000). Their influence on the secretion of oxygen radical metabolites appears to be modulated by the parallel production of •NO as recent studies have shown. A stimulation of arginine-enriched PMN cells leads not only to an increased production of superoxide anions, but also simultaneously to an increased metabolisation of intracellularly enriched arginine through the increased expression of the ''inducible'' •NO synthase (Huie and Padmaja, 1993). Since both metabolites show a good membrane as well as matrix mobility, and superoxide anions are also secreted via specific anion channels, they can certainly react with one another in the phagolysosomes as well as in the extragranulocytic matrix as various studies have confirmed (Fukuyama et al., 1996; Lee et al., 2000, 2002; Pryor and Squadrito 1995; Salvemini et al., 2003). As a result the increased superoxide anion secretion is not just converted more by superoxide dismutase to H_2O_2, but it is also ''scavenged'' in parallel by •NO (Squadrito and Pryor, 1995; Su et al., 1998). The transmutation of superoxide anions and •NO to peroxynitrite occurs approx. 3 1/2-times more rapidly than the enzymatically catalysed transformation to H_2O_2 (Beckman and Koppenol, 1996; Squadrito and Pryor, 1995, 1998). For these reasons various groups have postulated that peroxynitrite production might represent an essential metabolic pathway for •NO-mediated reactivity. The peroxynitrite, for example generated from leukocytes, is highly toxic and compared to HOCl appears to represent a key product for neutrophil-induced radical formation. In addition, peroxynitrite as well as the hydroxyl radical (OH) arising from it appear to be responsible for damage to tissues and cells associated with granulocyte activation in many diseases such as rheumatoid arthritis, Alzheimer's disease or certain forms of atherosclerosis (Arteel et al., 1999). These findings are particularly relevant for detection procedures based on chemiluminescence. Peroxynitrite, in the same way as HOCl, appears to be one of the main agents responsible for the observed effects (Nakano, 1998). Indirect evidence for a reaction between superoxide anions with •NO was also provided by our own research group. If the metabolic availability of a precursor metabolite is restricted by inhibiting •NO synthase, or by increasing the metabolisation of superoxide anions (e.g. through addition of superoxide dismutase), a significant reduction in peroxynitrite generation as well as an apparent ''activation'' of granulocyte oxygen radical formation can be observed. The putative ''activation'' might be explained by the reduced superoxide anion metabolisation with •NO to the benefit of transformation catalysed by the superoxide dismutase to H_2O_2 (Squadrito and Pryor, 1998, 1995). This was also shown from the results of arginine or ornithine application with parallel inhibition of •NO synthase activity. Here as well, a reversal of arginine-reduced superoxide anion generation and a further increase in hydrogen peroxide production could be verified. The influencing of oxygen radical formation induced by NOS inhibition might be associated not just with an increase, but even with a restriction of cytotoxic or also bactericidal activity of PMN cells, presumably as a direct result of the reduced peroxynitrite generation (Fukuyama et al., 1996; Lee et al., 2000, 2002). Pathophysiological correlates of these observations may lie for example in the activation of specific tissue protective mechanisms (such as selenium, metalloporphyrins, glutathione) to protect against or reduce granulocyte-mediated inflammatory processes. Clear findings and statements on this matter have not yet been reported in the literature. This can be explained on the one hand by the methodological problems encountered in recording the variety of oxygen free radicals secreted after PMN activation in the phagolysosome or indeed the extragranulocytic matrix, as well as the cytotoxic metabolites derived from them, and on the other by the fact that *in vitro* recorded cytotoxic and bactericidal functions can not be applied directly to the actual conditions in an inflamed matrix or the activities and signal transmission pathways of the tissues and cells that have migrated there (Azadniv et al., 2001). Regarding the free amino- and α-keto acid pools, an inhibition of •NO synthase, however, did not lead to any significant alterations. This is not surprising, since in earlier studies on PMN cells, significant alterations in cellular arginine content, e.g. through reduction of transmembranous arginine transport, could only be induced by application of extremely

high concentrations of specific •NO synthase inhibitors (e.g. >10 mM L-NAME; Stehr et al., 2004). In addition, the regulation of •NO production required for modulating cellular metabolic processes (modulation of inflammatory reaction) can be taken on at least in part by •NO synthase independent mechanisms, such as the reaction of arginine with H_2O_2 (Nagase et al., 1997). The varying regulation of •NO synthase isoenzymes and the highly variable •NO production in various cell and tissue compartments associated with that might explain the variety and apparent inconsistency of the •NO effects observed up until now (Armstrong, 2001). According to current hypotheses, •NO is not just a modulator of granulocytic metabolic processes and immune reactions; it also modulates the maintenance of other inflammatory reactions during an inflammatory event (e.g. endothelial cell or macrophage-mediated) as well as intercellular communication (Armstrong, 2001; Coleman, 2001). In this way •NO can significantly modulate essential cellular metabolic processes and enzyme functions in various cell and tissue types, such as the mitochondrial respiratory chain, ribonucleotide reductase (a key enzyme of protein biosynthesis), the superoxide anion generating enzyme NADPH oxidase, xanthine oxidase and also cellular •NO synthesis (Robbins and Singharajah, 2005). Previous findings have shown that •NO in PMN cells induces both biphasic and exposure-time-dependent effects (Lee et al., 2000, 2002). As part of an inflammatory event, the •NO from various cells and tissues (i.e. PMN, macrophages, endothelial cells, etc.) can reach the extracellular matrix where it can react with oxygen radical species (i.e. peroxynitrite, hydroxyl radicals, etc.). According to former study results (Lee et al., 2000, 2002; Pieper et al., 1994) low •NO as well as peroxynitrite concentrations should be able to induce PMN-activation with a raised production of superoxide anions. However, if formation and with that the •NO concentration in the extracellular matrix is so high that it can no longer be scavenged by the oxygen radicals produced (e.g. superoxide anions) and high peroxynitrite concentrations become evident, •NO as well as the reactive nitrogen metabolites derived from reactions with it may exert a direct influence on important granulocytic metabolic processes and immune reactions via a negative feedback mechanism. As the current findings were able to show, increasing and especially high extragranulocytic •NO concentrations, caused for example by application of •NO donors, induce not only a greater reduction of superoxide anion generation (due to inhibition of the NADPH oxidase complex), but also an •NO-dose-dependent inhibition of •NO synthase and reductions in β-glucuronidase secretion, leukotriene formation, degranulation activity, chemotactic properties and granulocytic adherence (Guzik et al., 2003; Sethi and Dikshit, 2000). This may represent a mechanism to protect body tissues and cells from the development of an uncontrollable, self-reinforcing immune response. A number of authors have postulated a critical balance between oxygen radical metabolites and •NO in the regulation of inflammatory processes, such as in the control of potential ''unwanted'' tissue damage (Bronte and Zanovello, 2005; Korhonen et al., 2005). In order to determine the potential effects of an inhibitory •NO effect on the intragranulocytic free amino- and α-keto acid pools and the immune functional parameters assessed here, an •NO donor was applied over the micromolar concentration range. As the results show, S-nitroso-N-acetyl-penicillamine (SNAP) induces clear reductions in granulocytic glutamine, glutamate, aspartate, arginine, ornithine, α-ketoglutarate and pyruvate levels. Apart from the inhibition of specific glutamine-dependent intracellular metabolic functions, a direct (extracellularly accumulated) •NO-mediated transmembranous amino acid loss also appears responsible for these findings (Bridges et al., 2001). In addition, the administration of SNAP led to a dose- and incubation time-dependent reduction in superoxide anion and hydrogen peroxide generation as well as a decrease in extragranulocytically secreted myeloperoxidase activity. The decrease in extracellularly secreted superoxide anion and hydrogen peroxide is most probably related both to an inhibition of cellular metabolic functions and the immune response itself, as well as the fact that •NO concentrations were artificially raised in the medium. This could lead to a reaction of •NO with oxygen radical metabolites potentially secreted in the matrix, such as superoxide anions, and significantly influence the detection of ROS produced by PMN in this experiment (Guzik et al., 2003; Cepinskas et al., 2002). This was also be shown when combining SNAP with arginine or ornithine. Regarding the assessed amino- and α-keto acids, administration of an •NO donor at micromolar concentrations also led to clear changes. Remarkably, the •NO-dependent modification of granulocytic amino- and α-keto acid pools was qualitatively quit similar to that induced by anaesthetics (i.e. midazolam, propofol, methohexital, etc.; Mühling et al., 2001b, 2002, 2005). Although this does not seem so surprising, the above-mentioned •NO-dependent functional modification of immunologically active cells appears to be based on an indirect influencing of primary metabolic functions. Several groups have shown that •NO can unleash concentration-dependent, not only cytoprotective, but also cytostatic and cytotoxic effects.

As such an •NO concentration-dependent inhibition of ribonucleotide reductase (DNA synthesis), ADP-ribosyltransferase and glyceraldehyde-3-phosphate-dehydrogenase (glycolysis and gluconeogenesis) and an •NO-mediated influencing of enzymes of the respiratory chain (ubiquinone reductase, cytochrome C oxidase) or the citric acid cycle (cis-aconitase) could be found. Many, but not all of these effects observed until now can be explained by changes in activity of soluble isoforms of the enzyme guanylate cyclase (Russwurm and Koesling, 2004; Blaise et al., 2005; Wanstall et al., 2005). A well known consequence is the modulation of ion channels such as the calcium ion transporter, an effect which underlies the •NO-dependent relaxation of vascular smooth muscle cells for example. The extent to which extracellularly enriched •NO influences granulocytic function as well as intracellular amino- and α-keto acid pools was revealed by combining SNAP with arginine or ornithine. Although arginine or ornithine administration did lead to an increase in intracellular arginine, ornithine and citrulline levels, the modulation of granulocytic metabolic processes by extracellularly supplemented •NO was, however, only so marked that this influence (compared to isolated SNAP application) led only to a reduction, and not a reversal of the findings.

For the first time the effects of L-NAME, SNAP, DFMO, arginine or ornithine as well as the combination of arginine or ornithine with L-NAME, SNAP or DFMO on intracellular free amino- and α-keto acid profiles and the immune function markers superoxide anion and hydrogen peroxide generation as well as released myeloperoxidase activity in neutrophils (PMN) have been investigated. Although the underlying mechanisms still remain unclear, we believe from our results that nitric oxide- as well as polyamine-dependent pathways are involved in the signal transmission of free radical molecule-, beneficial nutritional therapy- or maleficient pharmacological stress-induced alterations in PMN nutrient homeostasis. Relevant changes in intragranulocyte free amino- and α-keto acid homeostasis and metabolism, especially, may be one of the determinants in PMN nutrition that positively or negatively influences and modulate neutrophil host defence capability and immunocompetence.

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