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ORIGINAL ARTICLE

Effects of α-ketoglutarate on neutrophil intracellular amino and α-keto acid profiles and ROS production

Jörg Mühling · F. Tussing · K. A. Nickolaus · R. Matejec · M. Henrich · H. Harbach · M. Wolff · K. Weismüller · J. Engel · I. D. Welters · T. W. Langefeld · M. Fuchs · M. A. Weigand · M. C. Heidt

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Abstract The aim of this study was to determine the effects of α-ketoglutarate on neutrophil (PMN), free α-keto and amino-acid profiles as well as important reactive oxygen species (ROS) produced [superoxide anion (O_2^-), hydrogen peroxide (H_2O_2)] and released myeloperoxidase (MPO) acitivity. Exogenous α-ketoglutarate significantly increased PMN α-ketoglutarate, pyruvate, asparagine, glutamine, asparatate, glutamate, arginine, citrulline, alanine, glycine and serine in a dose as well as duration of exposure dependent manner. Additionally, in parallel with intracellular α-ketoglutarate changes, increases in O_2^- formation, H_2O_2-generation and MPO acitivity have also been observed. We therefore believe that α-ketoglutarate is important for affecting PMN "susceptible free amino- and α-keto acid pools" although important mechanisms and backgrounds are not yet completely explored. Moreover, our results also show very clearly that changes in intragranulocytic α-ketoglutarate levels are relevant metabolic determinants in PMN nutrition considerably influencing and modulating the magnitude and quality of the granulocytic host defense capability as well as production of ROS.

Keywords α-Ketoglutarate · Neutrophil · Amino acids · α-Keto acids · Immune function

J. Mühling (✉) · F. Tussing · K. A. Nickolaus · R. Matejec · M. Henrich · H. Harbach · M. Wolff · K. Weismüller · J. Engel · T. W. Langefeld · M. A. Weigand
Clinics of Anaesthesiology, Intensive Care Medicine and Pain Therapy, University Hospital Giessen and Marburg GmbH, Justus-Liebig-University Giessen, Rudolf-Buchheim-Strasse 7, 35385 Giessen, Germany
e-mail: joerg.muehling@chiru.med.uni-giessen.de

I. D. Welters
University Department of Anaesthesia, Royal Liverpool University Hospital, Liverpool, UK

M. Fuchs
Dr. Ing. Herbert Knauer GmbH, Berlin, Germany

M. C. Heidt
Department of Cardiovascular Surgery, University Hospital Giessen and Marburg, Justus-Liebig-University Giessen, Giessen, Germany

Introduction

The neutrophil (polymorphonuclear leukocyte, PMN) host defense mechanisms, their so-called "raison d'être", can be divided into different important steps (i.e. mediator release, phagocytosis, exocytosis, oxidative activiation) (Burg and Pillinger 2001; Kobayashi et al. 2001; Witko-Sarsat et al. 2000) and it is not surprising that each of the components of this sequence is triggered by signal transduction pathways which immediately transmit information to the metabolic machinery of the cell (Castell et al. 2004; Curi et al. 1997a, b; Cynober et al. 2007; Cynober 2002; Dhaliwal and Heyland 2005; Loi et al. 2005; Moinard et al. 1999; Mühling et al. 2002, 2005, 2006a, b; Roch-Arveiller et al. 1996). Increasing evidence suggests that α-ketoglutarate, the five-carbon backbone of glutamine and glutamate, especially, may have relevant immunonutritional value in the physiological state and bactericidal capacity of PMN (Castell et al. 2004; Curi et al. 1986, 1988, 1997a, b; Cynober et al. 2007, 1990; Cynober 1999, 2002; Loi et al. 2005, 2007; Moinard et al. 1999, 2000, 2002; Roch-Arveiller et al. 1996). Indeed, in all eukaryotic cells α-ketoglutarate, which can be produced by different pathways [i.e. oxidative deamination of glutamate (glutamate dehydrogenase), transamination, oxidative decarboxylation of isocitrate in the tricarboxylic

acid (TCA) cycle, etc.], plays a key role in cellular energetics and metabolism by functioning as an important source of respiratory cellular fuel and through its conversion into important metabolic precursors (Brown et al. 2004; Frei et al. 1975; Hausinger 2004; Lucia and Trznadel 1983; Willems et al. 1978). Once in the TCA cycle, α-ketoglutarate can be oxidized to CO_2 or serves as a precursor of numerous amino acids, synthetic metabolic intermediates as well as macromolecular synthesis (Lagranha et al. 2008a, b; Law et al. 1992; Loi et al. 2005, 2007). Indeed, on the basis of maximal enzyme activities, it has been suggested that the conversion of α-ketoglutarate to malate is catalysed by oxoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase and fumarase, malate to oxaloacetate by malate dehydrogenase, malate to pyruvate by NAD^+- or $NADP^+$-linked malate dehydrogenase (decarboxylating), oxaloacetate to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK), PEP to pyruvate by pyruvate kinase and pyruvate to aspartate by aspartate aminotransferase (Agam and Gutman 1972; Board et al. 1990; Curi et al. 1986, 1988, 1997a, b; Kirk and Heys 2003; Loi et al. 2005, 2007; Mühling et al. 2002, 2005, 2006a). Aspartate can in turn be converted (i.e. to asparagine) or may serve as a precursor or amine donor in pyrimidine and purine synthesis. Through pyruvate (i.e. via malate) α-ketoglutarate-derived carbon can be concerted to lactate or acetyl-CoA (i.e. used for the synthesis of fatty acids or phospholipids for cellular membranes) and its acetyl group can re-enter the TCA cycle, eventually leading to the complete respiratory oxidation of α-ketoglutarate carbons to CO_2 (Grimble 2001; Mitzkat et al. 1972; Mizuho et al. 1996; Moinard et al. 1999, 2000, 2002; Oehler and Roth 2003; Robinson et al. 1999; Willems et al. 1978). As mentioned above, another important steps in the formation or use of α-ketoglutarate are catalyzed by rapid and reversible transamination reactions (Curi et al. 1986, 1988, 1997a, b; Fauth et al. 1990, 1993; Mizuho et al. 1996; Moinard et al. 2000, 2002; Newsholme et al. 2003a, b; Newsholme 1994, 2001; Stjernholm et al. 1969). The glutamate arising from these reactions, especially, is the most abundant intracellular amino acid in neutrophils and therefore may play a key metabolic and synthetic role essential for cellular viability, since it can be very rapidly transformed into other essential cell substrates (Moreira et al. 2007; Mühling et al. 2002, 2005, 2006a, 2006b; Newsholme et al. 2003a, b; Newsholme 1994, 2001; Stjernholm et al. 1969).

The goals of this study are therefore to document the effects of α-ketoglutarate (regarding its role in PMN immunonutrition) on PMN free intracellular α-keto and amino acid concentrations and on important granulocytic immune functions (superoxide anion and hydrogen peroxide formation, activity of released myeloperoxidase).

Material and methods

The study was approved by the local ethics committee of the Justus Liebig University, Giessen. Ten men between 23 and 40 years (32 ± 5.1) with an average height of 179.5 cm (range 173–188) and weight of 80.2 kg (range 72–93) 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 h of fasting) with consideration of circadian variations.

α-Ketoglutarate

(1) To document any dose-dependent effects PMN were incubated with different α-ketoglutarate concentrations (0, 5, 10, 20, 50 and 100 μM) for 120 min. The selected α-ketoglutarate concentration corresponded to 0-, ½-, 1-, 2-, 5- and 10-fold the clinically achieved plasma concentrations (see Mühling et al. 2006a, b for physiological values).
(2) To examine if there is a critical duration of exposure is necessary to produce any significant effects PMN were incubated with α-ketoglutarate (100 μM) for 10, 60 or 120 min.

Solutions of α-ketoglutarate 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 α-ketoglutarate 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 PMN from whole blood

Precise details of our PMN-separation technique have been described previously (Mühling et al. 1999, 2003). This method allows a very rapid and selective enrichment of neutrophils while preserving high cellular viability and integrity from very small quantities of whole blood. Separation of PMN was accomplished using a cooled (4°C) Percoll®-gradient (Pharmacia, Uppsala, Sweden). Three 4 ml portions (Σ = 12 ml) of cooled whole blood from

each volunteer were overlaid onto previously prepared and precooled (4°C) 70/55% (in 0.9% NaCl) Percoll®-gradients before centrifugation at 350×*g* for 15 min at 4°C (Biofuge®, Heraeus, Hanau, Germany). This separates the PMN as a small layer between the erythrocyte and monocyte layers. The PMN were carefully removed from the sample and suspended in 10 ml cooled (4°C) phosphate buffered saline (PBS) stock buffer (diluted 1:10, v/v; 10× PBS stock buffer, without Ca^{2+}/Mg^{2+}, Gibco, Karlsruhe, Germany). After a second centrifugation step (350×*g* for 5 min at 4°C), the PBS buffer was discarded and the erythrocytes remaining in the sample were hypotonically lysed using 2 ml of cooled (4°C) distilled water (Pharmacia, Uppsala, Sweden). After 20 s the PMN fraction was immediately brought back to isotonicity by the addition of 1 ml of 2.7% NaCl (Merck, Darmstadt, Germany) at 4°C and resuspended by adding 10 ml of diluted stock PBS buffer. After a third centrifugation step (350×*g* for 5 min at 4°C) the PBS buffer was discarded and the PMN fraction again resuspended (200 µl PBS buffer). Subsequently, all PMN fractions were combined and two aliquots of resuspended sample were removed for microscopy. On average, the cell fractionation procedure lasted 34 ± 4 min. Immediately after preparation, the extracted PMN samples were frozen at −80°C before lyophilization (freeze dryer CIT-2®, Heraeus, Hanau, Germany). These conditions allowed for a PMN lysis which was not chemically mediated and guaranteed longer analyte stability during extended storage of the sample. Samples prepared in this manner were stored at −80°C until analyzed within a period not exceeding four weeks. The purity, determined in duplicate in the first aliquot by dying with "Türk's Solution®" (Merck) and viability, determined in the second aliquot by exclusion of "Trypan Blue®" (Merck) were examined and verified by light microscopy (Zeiss, Oberkochen, Germany). Cell yields were determined at the same time that viability was measured, samples with a PMN purity and viability < 96% were discarded. In parallel, plasma samples (100 µl) were separated, lyophilized and storaged using known techniques.

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 acid and α-keto acid analysis, specially developed and precisely validated in our institute for this purpose. Moreover, the coefficients of variations for both the method reproducibility and reproducibilities of the retention times were also within normal ranges (for details see Mühling et al. 1999, 2003). PMN amino acid concentrations are given in 10^{-16} moles per neutrophil-cell, PMN α-keto acid concentrations are given in 10^{-17} moles per neutrophil-cell.

Preparation of derivatization reagent

For the fluorescence labeling of the α-keto acids, we used *o*-phenylenediamine (OPD, Sigma, Deisenhofen, Germany). Since oxidation of OPD influences the results in a negative way (the oxidized reagent causes variation in the fluorescence intensity) the brown powder must be re-crystallized prior to use. Although the amount of reactive OPD is less when using the oxidized form of the reagent, this re-crystallization procedure is necessary even when starting with the originally supplied substance. The *o*-phenylenediamine was dissolved in heptane at a temperature of 100–120°C (oil bath, Merck) and the heptane subsequently evaporated in a rotary evaporator (Merck). This procedure yielded a white powder after drying. With storage under N_2 (Sigma, Deisenhofen, Germany) and at 4°C in a dark bottle, the dry substance is useable for several months. For each batch of analyses, the OPD reagent must be freshly prepared. For each sample, 5 mg of OPD was dissolved in 5 ml of 3 M HCl (Sigma) and 10 µl of 2-mercaptoethanol (Sigma) was added to yield OPD-HCl-ME. This reagent solution was stable for several hours without loss in sensitivity [13, 27].

Standard samples and precolumn derivatization procedure

Analytically pure α-keto acids (Sigma) were dissolved in distilled H_2O (Merck) containing 4% human serum albumin (Merck), immediately lyophilized and stored at −80°C. The lyophilizates (PMN, plasma and standard samples) were solubilized in 250 µl of pure methanol (Mallinckrodt Baker B·V., Deventer, Holland). The methanol also contained the α-keto acid, α-ketovalerate (KV; Sigma) as an HPLC internal standard. KV is a non-physiological α-keto acid. After a 3-min incubation and a 3-min centrifugation step (3,000×*g*, Rotixa/KS®, Tuttlingen, Germany), 200 µl of the extracts were dried under N_2 (10 min, 20°C, Messer, Griesheim, Germany). The OPD-HCl-ME reagent (5 ml) was then added, and the samples were incubated for 60 min at 80°C. The derivatization was stopped after exactly 60 min by cooling for 15 min in ice water. Ethyl acetate (2 ml, Sigma) was added to the samples and mixed for 7 min in an rotary mixer (Merck) to extract the α-keto acids. After extraction, the top ethyl acetate layer was then transferred to a glass vial (2-CRV®, Chromacoll, Trumbull, USA). This procedure was repeated twice for each sample. The combined etylacetate portions were dried under N_2 (30 min), re-solubilized in 120 µl of methanol and 50 µl of this mixture was injected onto the HPLC column.

Fluorescence high-performance liquid chromatography

The fluorescence high-performance liquid chromatography system (F-HPLC) consisted of a pump with a controller for gradient programming (600 E®, Waters, Milford, MA, USA) and a programmable autosampler (Triathlon®, Spark, Netherlands) with a Rheodyne injection valve and a 100 µl sample loop (AS 300®, Sunchrom, Friedrichsdorf, Germany). A Nova-Pak®, 300 × 3.9 mm i.d., RP-C-18, 60Å, 4 µm (Waters) analytical column was used for the separation. Column temperatures were maintained at 35°C using a column oven (Knauer, Berlin, Germany). The column eluent was monitored using a fluorescence spectrophotometer (RF-530®, Shimadzu, Kyoto, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 415 nm. Data recording and evaluation was performed using computer integration software (EuroChrom 2000®, Knauer, Berlin, Germany). The linear calibration curves were constructed based on area ratios of the standard (St) to the sample (S) chromatogramms [($\text{area}_{\text{keto acid}-\text{St}}/\text{area}_{\text{internal standard}-\text{St}}$] × amount or concentration of keto acids injected = calculation factor (CF); [$\text{area}_{\text{keto acid}-\text{S}}/\text{area}_{\text{internal standard}-\text{S}}$) × CF = final result]. The flow rate was maintained at 1.0 ml/min throughout. For the gradient program and solvents, automatically degassed using a three-channel degasser (Knauer, Berlin, Germany) see Mühling et al. (1999, 2003).

Superoxide anion production

Superoxide anion and hydrogen peroxide production as well as activitiy of released myeloperoxidase were determined photometrically using modifications of known methods validated in our institute for this purpose (for further details see Mühling et al. 2002, 2005, 2006a, 2006b). Superoxide anion production was measured by reduction of cytochrome C. 100 mg of cytochrome C (type IV, Sigma, Deisenhofen, Germany) which was dissolved in 30 ml PBS®-glucose buffer. The solution was portioned and frozen at −20°C. Opsonized zymosan (Sigma, Deisenhofen, Germany) was used to stimulate PMN. It was produced by incubating 100 mg zymosan with 6 ml pool serum for 30 min at 37°C. After washing with saline and centrifuging at 350×*g* (10 min) opsonized zymosan was re-suspended in 10 ml PBS®-glucose buffer, portioned and frozen at −20°C. Whole blood was incubated either with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested for 2 h at 37°C. The PMN were then isolated using a modification of our PMN-separation technique (as mentioned above). After stepwise (15 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 resuspen-ded by adding diluted PBS® (Gibco, Karlsruhe, Germany) stock buffer. After 7 ml PBS® stock buffer had been administered, the tube was centrifuged at 350×*g* for 5 min (20°C). The supernatant was decanted. Samples with a PMN purity < 96% and those with more than 4% dead cells were discarded. The PMN concentration required in each case was adjusted by adding PBS containing 9.99 g glucose (Merck, Darmstadt, Germany). After PMN isolation, 500 µl zymosan, 150 µl pool serum, 250 µl cytochrom C and 500 µl isolated PMN suspension (0.8 × 10^6/ml) and again diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested, were poured into a test tube. A preparation containing 500 µL buffer instead of zymosan was used as a zero adjustment. After further incubation for 15 min at 37°C the reaction was stopped by putting the test tube into ice water. After centrifugation (350×*g*; 5 min, 4°C) extinction of the supernatant was measured photometri-cally (546 nm; Digitalphotometer 6114S®; Eppendorf, Germany). The amount of superoxide anion produced resulted from the extinction coefficient of cytochrome C according to the law of Lambert and Beer. All control probes obtained for standard curves have been prepared, incubated and measured identically.

Hydrogen peroxide production

Hydrogen peroxide production was also determined photometrically. The method based on horse radish peroxidase catalysed by oxidation of phenol red by hydrogen peroxide. Phenol red (Sigma, Deisenhofen, Germany) and horse radish peroxidase (type II, Sigma, Deisenhofen, Germany) were added to PMN which had been stimulated by opsonized zymosan. Phenol red was dissolved in double-destilled water (10 g/l). Horse radish peroxidase was dissolved in PBS®-glucose buffer (5 g/l). After incubation of whole blood with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested for 2 h at 37°C PMN were isolated as described above. Isolated PMN were stimulated by opsonized zymosan (Sigma, Deisenhofen, Germany). The final test preparation consisted of 500 µl zymosan, 125 µL pool serum, 12.5 µl horse radish peroxidase, 12.5 µl phenol red, 12.5 µl sodium azide (200 mmol/l/l; Merck, Darmstadt, Germany), 500 µl PMN suspension (2 × 10^6 PMN-cells/ml) and again diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine. After incubation for 15 min (37°C), the test preparation was centrifuged for 5 min (350×*g*; 4°C). Subsequent to adding 25 µL sodium hydroxide solution (1.0 normal, Merck, Darmstadt, Germany), the extinction was measured photometrically at 623 nm. All control probes obtained for standard curves have been prepared, incubated and measured identically.

Activitiy of released myeloperoxidase

Activitiy of released myeloperoxidase was also determined photometrically. About 1 mmol/l 2.2′-azino-di-(3-ethyl-benzthiazoline) sulphonic acid (ABTS, Sigma, Deisenhofen, Germany) was dissolved in 0.1 mol/l citrate buffer (Behring, Marburg, Germany; pH 7.4). After incubation of whole blood with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine to be tested for 2 h at 37°C, 100 µl isolated PMN suspension (2×10^6/ml) was incubated with 0.5 µg cytochalasin B (Sigma, Deisenhofen, Germany) and again with diazepam, L-alanyl-L-glutamine or diazepam combined with L-alanyl-L-glutamine (5 min; 37°C). After adding 100 µL opsonized zymosan and supplementing in order to keep the concentration constant, the preparation was incubated again for 10 min (37°C). Then 1 ml ATBS solution was added. After centrifuging (700×*g*, 5 min, 20°C) 1 ml of supernatant was removed and mixed with 1 µl hydroxide peroxide solution (30%; Merck, Darmstadt, Germany) and extinction was measured photometrically (405 nm).

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 formation, hydrogen peroxide generation as well as activity of released myeloperoxidase obtained in the control cells were within normal physiological ranges (see Mühling et al. 1999, 2003) (Table 1). Intracellular α-ketoglutarate levels amounted on average to 1.37×10^{-17} moles per neutrophil (PMN) cell [≈37 µmol/l PMN cell volume (NCV); plasma: 8.2 ± 3.1 µmol/l]. The intracellular pyruvate content at almost 5.94×10^{-17} mol per PMN cell (≈163 µmol/l NCV; plasma: 106.7 ± 36 µmol/l) was approximately 4.3 times higher.

Table 1 Effects of α-ketoglutarate (KG; 100 µM) incubated with whole blood for 10, 60 and 120 min on important free intracellular amino acid (10^{-16} mol per PMN-cell; mean ± SD; $n = 10$), α-ketoglutarate and pyruvate concentrations in PMN (10^{-17} Mol per PMN-cell; mean ± SD; $n = 10$) as well as on PMN superoxide anion production [O_2^-; fmol/(PMN × min)$^{-1}$], hydrogen peroxide formation [H_2O_2; fmol/(PMN × min)$^{-1}$] and myeloperoxidase activity (MPO; units/l supernatant); (mean ± SD; $n = 10$)

	10 min		60 min		120 min	
	Mean	SD	Mean	SD	Mean	SD
Control						
Asparagine	0.39	0.08	0.40	0.08	0.41	0.09
Glutamine	2.91	0.68	3.05	0.78	3.18	0.88
Aspartate	2.67	0.69	2.77	0.51	2.80	0.63
Glutamate	6.04	1.08	6.41	1.42	5.88	1.47
Ornithine	0.35	0.07	0.33	0.07	0.36	0.08
Arginine	0.46	0.08	0.44	0.08	0.47	0.09
Citrulline	0.13	0.03	0.12	0.03	0.13	0.03
Serine	2.25	0.40	2.31	0.44	2.27	0.49
Glycine	2.37	0.39	2.46	0.51	2.41	0.57
Alanine	1.85	0.38	1.83	0.42	1.82	0.46
α-Ketoglutarate	1.37	0.40	1.43	0.32	1.50	0.45
Pyruvate	5.94	1.47	6.47	1.81	6.17	1.32
O_2^-	3.305	0.542	3.227	0.602	3.018	0.714
H_2O_2	1.186	0.233	1.132	0.265	1.061	0.270
MPO	0.558	0.147	0.523	0.152	0.481	0.149
KG (100 µM)						
Asparagine	0.38	0.06	0.48*#	0.08	0.61*#	0.12
Glutamine	3.07	0.70	4.34*#	0.82	5.49*#	1.42
Aspartate	2.55	0.59	3.69*#	0.90	4.53*#	0.97
Glutamate	6.26	1.25	9.88*#	2.47	12.73*#	3.18
Ornithine	0.36	0.09	0.43*	0.09	0.51*#	0.12
Arginine	0.49	0.10	0.57*	0.13	0.67*#	0.13
Citrulline	0.12	0.03	0.13	0.04	0.16	0.03
Serine	2.10	0.41	2.41	0.55	2.86*#	0.56
Glycine	2.31	0.43	2.69	0.57	3.16*#	0.77
Alanine	1.92	0.43	2.36*	0.46	2.90*#	0.89
α-Ketoglutarate	1.28	0.45	2.57*#	0.68	3.95*#	1.45
Pyruvate	6.29	1.87	9.86*#	2.63	12.47*#	3.76
O_2^-	3.944	0.779	6.167*#	1.651	8.225*#	2.364
H_2O_2	1.372	0.338	2.365*#	0.533	3.255*#	0.990
MPO	0.620	0.204	0.843*	0.239	1.143*#	0.436

* $P \leq 0.05$ versus control values, # $P \leq 0.05$ versus 10 min

The intragranulocytic amino acids glutamine, glutamate and alanine associated with α-ketoglutarate and pyruvate metabolism could also be exactly quantified: their average concentrations at 2.91×10^{-16} mol per PMN cell (glutamine) (≈799 µmol/l NCV; plasma: 528 ± 166 µmol/l), 6.04×10^{-16} mol per PMN cell (glutamate) (≈1.659 µmol/l NCV; plasma: 32 ± 8 µmol/l) and 1.85×10^{-16} mol

per PMN cell (alanine) (≈508 µmol/l NCV; plasma: 346 ± 93 µmol/l), respectively, were on average more than an order of magnitude higher. Further interesting findings were also obtained for asparagine, aspartate, arginine, ornithine, serine and glycine. Their intracellular contents were on average 0.39 × 10^{-16} mol (asparagine) (≈107 µmol/l NCV; plasma: 56 ± 15 µmol/l), 2.67 × 10^{-16} mol (aspartate) (≈734 µmol/l NCV; plasma: 12 ± 3 µmol/l), 0.46 × 10^{-16} mol (arginine) (≈126 µmol/l NCV; plasma: 81 ± 19 µmol/l), 0.35 × 10^{-16} mol (ornithine) (≈96 µmol/l NCV; plasma: 44 ± 19 µmol/l), 2.25 × 10^{-16} mol (serine) (≈618 µmol/l NCV; plasma: 125 ± 32 µmol/l) and 2.37 × 10^{-16} mol (glycine) (≈651 µmol/l NCV; plasma: 244 ± 56 µmol/l) per PMN cell, respectively. However, the composition of these free amino acid and α-keto acid pools does not appear to be arbitrary in any way. When comparing the intra versus the extracellular concentration gradient (i:e) the results painted a very different picture: for pyruvate (i:e: 1.52), glutamine (i:e: 1.51), alanine (i:e: 1.47), arginine (i:e: 1.55), serine (i:e: 4.94) and glycine (i:e: 2.66) not only high plasma but also intracellular concentrations have been found, while α-ketoglutarate (i:e: 4.5), glutamate (i:e: 51.8), aspartate (i:e: 61.2), asparagine (i:e: 1.91), ornithine (i:e: 2.2) with low plasma concentrations apparently accumulated within the neutrophils.

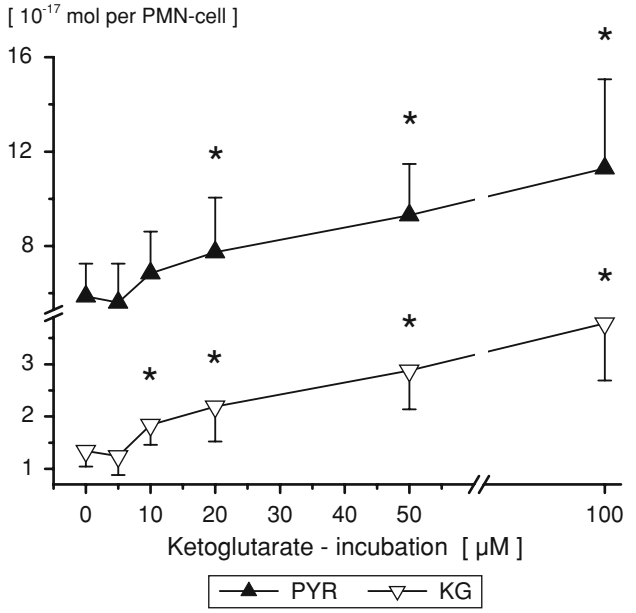

Fig. 1 Free intracellular α-ketoglutarate (*KG*) and pyruvate (*PYR*) concentrations in PMN-cells following α-ketoglutarate incubation (0, 5, 10, 20, 50, 100 µM; 120 min) of whole blood in vitro. PMN amino acid concentrations are given in 10^{-17} Mol per PMN-cell (mean ± SD; $n = 10$). * $P \leq 0.05$ versus control values

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

About 100 µM α-ketoglutarate significantly increased α-ketoglutarate and pyruvate profiles in a duration of exposure dependent manner (PMN incubation for ≥60 min; Table 1). Following low α-ketoglutarate doses (5 µM, PMN incubation for 120 min) concentrations of free intracellular α-keto acids remained uneffected. In the presence of higher α-ketoglutarate concentrations (PMN incubation for 120 min) significant dose-dependet increases in PMN α-ketoglutarate (≥10 µM) and pyruvate (≥20 µM) were obeserved (Fig. 1). PMN free α-ketobutyrate, α-ketoisovaleriate, α-ketoisocapronate, p-hydroxyphenylpyruvate and α-keto-β-methylvaleriate profiles remained uneffected.

Effects of α-ketoglutarate on free amino acid pool in PMN

Concentrations of free intracellular amino acids were uneffected by 5 µM α-ketoglutarate (PMN incubation for 120 min), respectively. Following higher α-ketoglutarate concentrations (PMN incubation for 120 min), significant dose-dependet increases increases in PMN glutamate (≥10 µM; Fig. 2), asparagine, glutamine, aspartate, alanine (≥20 µM; Fig. 2), ornithine and arginine (≥50 µM; Fig. 3), as well as in serine and glycine (≥100 µM; Fig. 4), have been observed. Moreover, 100 µM α-ketoglutarate (PMN incubation for ≥60 min) significantly increased PMN glutamate, glutamine, asparagine, aspartate, alanine, ornithine, arginine, citrulline, glycine and serine in duration of exposure dependent manner (Table 1). PMN free lysine, isoleucine, leucine, valine, methionine, taurine, hypotaurine, threonine, a-aminobutyrate, tyrosine, tryptophane, phenylalanine and histidine profiles remained uneffected.

Effects of α-ketoglutarate on oxidative response and myeloperoxidase activity

All PMN immune functions tested were uneffected up to 5 µM α-ketoglutarate. In the presence of higher α-ketoglutarate supplementation superoxide anion generation (≥10 µM), hydrogen peroxide formation (≥10 µM) and MPO activity (≥20 µM) increased significantly in a dose-dependent manner (PMN incubation for 120 min; Fig. 5). Relevant changes in PMN immune functions tested mainly occurred with PMN incubation for 60 min or longer (Table 1).

Discussion

Exogenous α-ketoglutarate incubated in whole blood siginificantly increased PMN intracellular α-ketoglutarate content

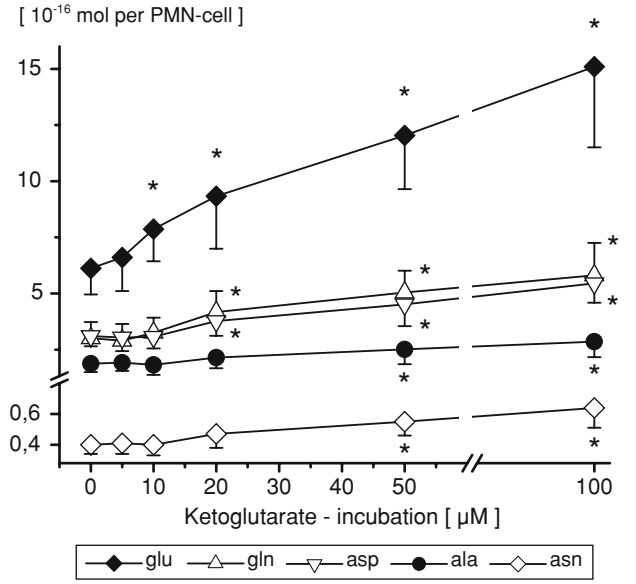

Fig. 2 Free intracellular glutamine (*gln*), glutamate (*glu*), asparagine (*asn*), aspartate (*asp*), and alanine (*ala*) concentrations in PMN-cells following α-ketoglutarate incubation (0, 5, 10, 20, 50, 100 µM; 120 min) of whole blood in vitro. PMN amino acid concentrations are given in 10^{-16} mol per PMN-cell (mean ± SD; $n = 10$). * $P \leq 0.05$ versus control values

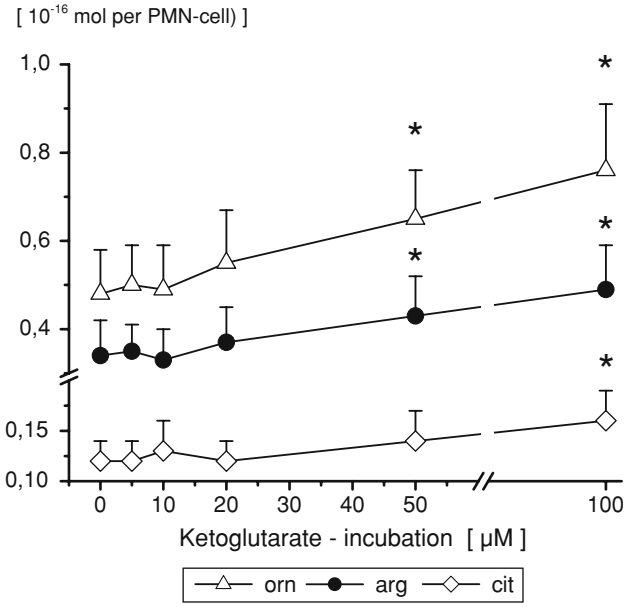

Fig. 3 Free intracellular ornithine (*orn*), arginine (*arg*) and citrulline (*cit*) concentrations in PMN-cells following α-ketoglutarate incubation (0, 5, 10, 20, 50, 100 µM; 120 min) of whole blood in vitro. PMN amino acid concentrations are given in 10^{-16} mol per PMN-cell (mean ± SD; $n = 10$). * $P \leq 0.05$ versus control values

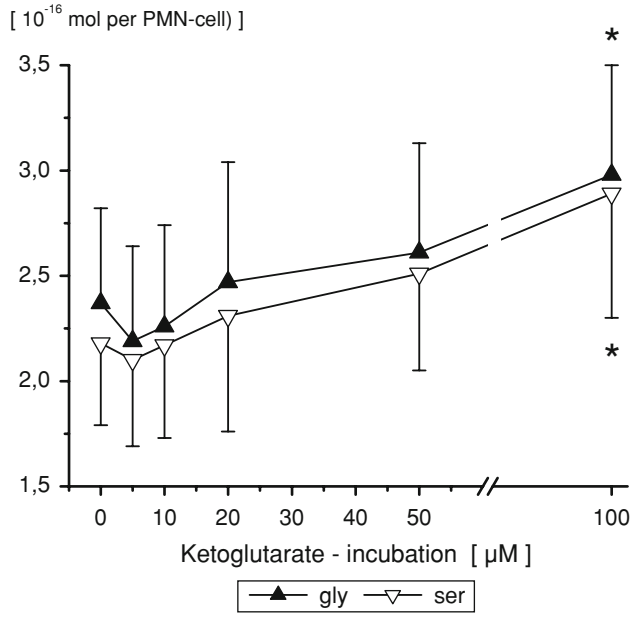

Fig. 4 Free intracellular glycine (*gly*) and serine (*ser*) concentrations in PMN-cells following α-ketoglutarate incubation (0, 5, 10, 20, 50, 100 µM; 120 min) of whole blood in vitro. PMN amino acid concentrations are given in 10^{-16} mol per PMN-cell (mean ± SD; $n = 10$). * $P \leq 0.05$ versus control values

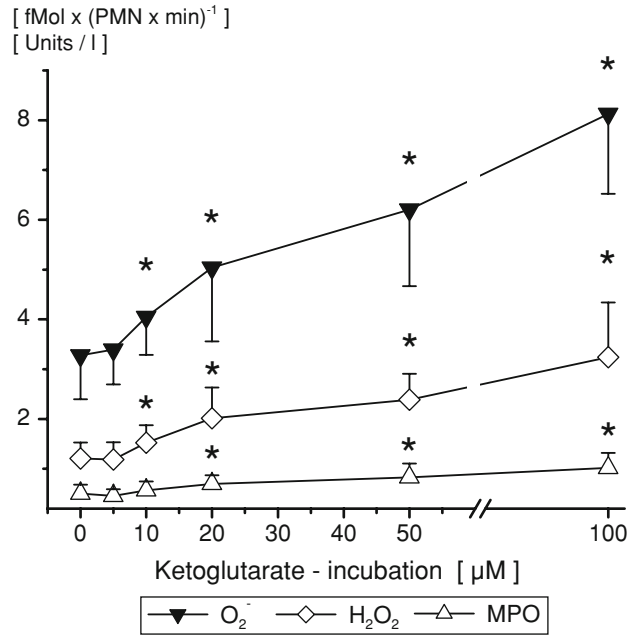

Fig. 5 Effects of different α-ketoglutarate concentrations (0, 5, 10, 20, 50, 100 µM) incubated with whole blood for 120 min on PMN superoxide anion production [O_2^-; fmol/(PMN × min)$^{-1}$], hydrogen peroxide formation [H_2O_2; fmol/(PMN × min)$^{-1}$] and myeloperoxidase activity (MPO; units/l supernatant); (mean ± SD; $n = 10$). * $P \leq 0.05$ versus control values

in a dose as well as duration of exposure dependent manner. This is not surprising taking into consideration that α-ketoglutarate can enter various eukaryotic cells by different active organic anion transporter mechanisms (i.e. different Na^+-dicarboxylate cotransporter proteins have been described) or simply by diffusion (Chen et al. 1998; Law et al.

1992; Pajor 1999). Interestingly, intracellular α-ketoglutarate also seems to be a major determinant for the efficacy of cellular organic anion transport, and events that alter internal α-ketoglutarate concentration, gradient, or both are poised to exert significant control over organic anion secretion (Burckhardt and Burckhardt 2003). Moreover, referring to our findings we also note an relevant role of α-ketoglutarate as an important intracellular substrate of various α-keto and amino acid pathways, because when intracellular α-ketoglutarate increases, the concentrations of pyruvate as well as glutamine, glutamate, asparagine, aspartate, alanine, arginine, ornithine, glycine and serine were also significantly elevated in a dose as well as duration of exposure dependent manner. Although our study does not allow one to infer whether the effects were direct (i.e. intracellular α-ketoglutarate conversion) or metabolic changes induced by α-ketoglutarate incubation of whole blood (i.e. uptake of important substrates by neutrophils produced from other blood cells), however, our findings reproducibly showed that α-ketoglutarate supplemented to whole blood significantly altered important further neutrophil free amino and α-keto acids (Curi et al. 1986, 1988; Cynober et al. 2007, 1990; Cynober 1999, 2002; Loi et al. 2007; Moinard et al. 2000, 2002). Moreover, the pattern of PMN amino and α-keto acid changes following α-ketoglutarate incubation favour the hypothesis that increases in neutrophil free α-ketoglutarate concentrations, similar to relevant immunonutritional effects induced by alanylglutamine, ornithine or arginine, are followed by α-ketoglutarate conversion into amino and α-keto acid derivatives (see Moinard et al. 2000, 2002; Mühling et al. 2002, 2005, 2006a, 2006b; Newsholme et al. 2003a, b; Newsholme 2001). From our results obtained until now we therefore suggest that α-ketoglutarate may act as substrate for subsequent intracellular metabolization, and this process also occurs within PMN cells. Enzymes required for this are certainly present in PMN cells as others have shown (i.e. glutamine-synthetase, glutamate-cysteine-ligase, glutamate-dehydrogenase as well as reversible transaminase reactions with an equilibrium constant in leukocytes at approximately 1, etc.) (Curi et al. 1986, 1988, 1997a, b; Fauth et al. 1990, 1993; Mizuho et al. 1996; Newsholme 1994; Stjernholm et al. 1969). Concerning to former findings we also believe, that the indirect availability of α-ketoglutarate in the form of glutamate, glutamine, alanine, asparagine, aspartate, arginine, ornithine, serine or glycin may emphazise the fundamental importance of this molecule in restoring pathophysiological depleted amino and α-keto acid pools (Castell et al. 2004; Curi et al. 1986, 1988, 1997a, b; Cynober et al. 2007, 1990; Cynober 1999, 2002; Loi et al. 2007, 2005; Moinard et al. 1999, 2000, 2002; Roch-Arveiller et al. 1996). Thus regulation of glutamine and glutamate metabolism (for example used for glutathione, proline or purine and pyrimidine synthesis, export of glutamate in exchange for import of cystine and following conversion to cysteine, synthesis of glucosamines or NAD^+, etc.), the formation of arginine, ornithine or aspartate via oxalacetate as a substrate for the enzymes of the urea cycle or formation of phosphoserine and serine and ultimately (with PMN activation) the synthesis of ·NO can all be modulated (Agam and Gutman 1972; Board et al. 1990; Castell et al. 2004; Curi et al. 1997a, b; Dhaliwal and Heyland 2005; Hausinger 2004; Moinard et al. 2002; Mühling et al. 2002, 2006b; O'Dowd and Newsholme 1997). Moreover, α-ketoglutarate can directly be shunted into the tricarboxylic acid cycle and oxidatively decarboxylated to succinyl-CoA by the α-ketoglutarate dehydrogenase complex and therefore serves as a respiratory fuel source and precursor of many synthetic intermediates or metabolic pathways (Hausinger 2004; Oehler and Roth 2003; Robinson et al. 1999 Roch-Arveiller et al. 1996). Once in the tricarboxylic acid cycle α-ketoglutarate, for example in the form of aspartate (via oxalacetate), becomes also indirectly available as a substrate for the enzymes of the urea cycle (Curi et al. 1988, 1997a, b; Huynh 2005). Moreover, α-ketoglutarate can be converted to malate and subsequently to pyruvate, the starting point for the "de novo synthesis" of alanine, serine, glycine as well as glucose (key enzyme for gluconeogenesis fructose-1,6-bisphosphatase had also been found in leukocytes) (Agam and Gutman 1972; Curi et al. 1986, 1988; Mitzkat et al. 1972; Newsholme 1994; Schrijver and Hommes 1975; Willems et al. 1978). Changes in intracellular α-ketoglutarate concentrations can also modulate the formation of arginine and ornithine and with that the synthesis of ·NO required for neutrophil activation (Coleman 2001; Moinard et al. 2002; O'O'Dowd and Newsholme 1997; Tan et al. 2008; Wu et al. 2008).

Concerning our results we therefore postulate that α-ketoglutarate, alongside glutamine, glutamate or glucose is an important metabolic precursor also for neutrophils and may also play a key metabolic role since it can be very rapidly transformed into other essential cell substrates as a so-called "intracellular turntable" for amino and α-keto acid metabolism. Interestingly, an overall view to the "phenotype" of all α-ketoglutarate-results presented till today suggest that the major immunonutritional value for neutrophils following α-ketoglutarate incubation may depend from the relevant rises in intracellular glutamine and glutamate levels, especially (Alpers 2006; Lagranha et al. 2008a, b; Li et al. 2007). Interestingly, the α-ketoglutarate-dependence of cellular function in neutrophils seems to be comparable with that of other leukocytic cells (Cynober et al. 2007; Cynober 2002; Engel et al. 2008; Loi et al. 2007 and 2005; Moinard et al. 1999, 2000, 2002; Wang et al. 2008). Indeed, recent observations showed that

artificial reductions in extracellular glutamine and glutamate concentrations, for example induced by intracellular inhibition of directly glutamine-dependent metabolic processes and enzymes or followed by application of specific glutamine analogues, are associated with significant impairments of neutrophil immunological function and metabolic activities (i.e. phagocytosis, bactericidal activity, oxygen radical production, secretion and degranulation of immunologically active enzymes and cytokines, etc.) (Das et al. 2007; Matés et al. 2006, 2008; Mühling et al. 2005, 2006a; Vermeulen et al. 2007). Moreover, inhibition of glutamine or glutamate metabolism also severely influenced further cellular metabolism to their metabolically-physiologically active α-keto acids. The glutamate and glutamine arising, for example formed from α-ketoglutarate, occupies key positions, since they can be very rapidly converted into other essential cell substrates (i.e. via reversible transaminations involving the release of NH_3). For example, alanine aminotransferase transfers the amino group of glutamate to pyruvate resulting in the production of alanine or aspartate aminotransferase produces aspartate where the amino group is bound to oxalacetate (Hausinger 2004; Kobayashi et al. 2005; Witko-Sarsat et al., 2000; Venizelos and Hagenfeldt 1985; Yeh et al. 2006). Other reaction pathway involving intracellular glutamine transformation to arginine and ornithine might also be catalyzed by the enzymes pyrroline-5-carboxylate synthase, ornithine aminotransferase, ornithine carbamyltransferase and carbamylphosphate synthase (Igarashi et al. 2000; Murphy and Newsholme 1998; Tan et al. 2008; Wu et al. 2004). Especially worthy of mention here is the condensation to the tripeptide glutathione (γ-Glu-Cys-Gly) (Matés et al. 2006, 2008; Sahoo et al. 1998; Wang et al. 2008). This reaction pathway is of high importance especially for leukocytic cells such as neutrophils, but an essential task of a intracellularly reduced glutathione is to protect the cell against attack by reactive oxygen species such as the highly membrane permeable H_2O_2 (Carr and Winterbourn 1997). Disorders in intragranulocytic glutathione metabolism, as in certain forms of inborn or acquired reductions in NADPH-dependent glutathione-reductase activity, are not of an insignificant pathophysiological or clinical importance (Engel et al. 2008; Johnson et al. 2006).

But the metabolization of α-ketoglutarate by the TCA cycle does not just supply intracellular carbon and nitrogen precursors for the above-mentioned metabolic pathways, since it also provides NADPH (Newsholme et al. 2003a, b; Newsholme 2001; Board et al. 1990; Frei et al. 1975; Witko-Sarsat et al. 2000). 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 neutrophils 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 neutrophilic immune defense (Burg and Pillinger 2001; Dahlgren and Karlsson 1999; Frei et al. 1975; Kobayashi et al. 2001). For this reason it is not surprising that the increase in α-ketoglutarate occurring upon application is also associated with a large increase in intracellular redox systems which improves PMN immune function. Consequently, we suggest that nutritional or pharmacological regimens which enhance the supply of α-ketoglutarate to PMN may have considerable value in modulating essential neutrophil functions, but the migration of immunonutritively metabolically and functionally improved neutrophils into an inflammatorily damaged extravascular matrix may also wreak additional serious damage to body cells or organ systems; especially when the granulocytic secretory and degranulation processes can not be balanced by a similarly nutritionally promoted system of cellular- and matrix-dependent protective functions (i.e. as the result of oedema formation, raised substrate consumption, etc., which pathophysiologically alteres or impaires the systemic supply of the inflammation region itself) (Alpers 2006; Bracco 2005; Dechelotte et al. 2006; Moreira et al. 2007; Vermeulen et al. 2007). Here, a major risk of an additional granulocyte-mediated injury to all cells and tissue structures is present there (Yeh 2006; Nussler et al. 1999; Parry-Billings et al. 1990). Various findings suggest, that in different cells at higher H_2O_2 concentrations inhibition of α-ketoglutarate dehydrogenase (α-KGDH) limits the amount of NADH available for the respiratory chain which emphasize the importance of (α-KGDH) in impaired mitochondrial function under oxidative stress (Sheu and Blass 1999; Robinson et al. 1999). For example enhanced free radical generation as well as loss of mitochondrial respiration and α-KGDH activity have been observed in Parkinson's and Alzheimer's diseases as well as in cardiac ischemia/reperfusion injury (Shi et al. 2008). Therefore, the α-ketoglutarate-mediated functional maintenance or even promotion in the granulocytic O_2^--H_2O_2-MPO system and immune functions in critically ill patients, however, might paradoxically induce injury to the body's own cells and tissue and in so doing aggravate the course of the disease inadvertently (Brandon 2004; Buchmann 2003, 2001; Castell et al. 2004; Dhaliwal and Heyland 2005 Lagranha et al. 2008a, b).

Referring to our results, it seems clear that α-ketoglutarate fulfills the criteria for a potent molecule in the regulation of the dynamic α-keto and amino acid pools as well as in modulation of PMN host defense mechanisms and immunoregulation. However, further urgent research is necessary to clarify α-ketoglutarate's sole role in vivo therapeutic immunonutritional properties.

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