Pyruvate: immunonutritional effects on neutrophil intracellular amino or alpha-keto acid profiles and reactive oxygen species production

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Abstract For the first time the immunonutritional role of pyruvate on neutrophils (PMN), free α-keto and amino acid profiles, important reactive oxygen species (ROS) produced [superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$)] as well as released myeloperoxidase (MPO) activity has been investigated. Exogenous pyruvate significantly increased PMN pyruvate, α-ketoglutarate, asparagine, glutamine, aspartate, glutamate, arginine, citrulline, alanine, glycine and serine in a dose as well as duration of exposure dependent manner. Moreover, increases in $O_2^-$ formation, $H_2O_2$-generation and MPO activity in parallel with intracellular pyruvate changes have also been detected. Regarding the interesting findings presented here we believe, that pyruvate fulfills considerably the criteria for a potent immunonutritional molecule in the regulation of the PMN dynamic α-keto and amino acid pools. Moreover it also plays an important role in parallel modulation of the granulocyte-dependent innate immune regulation. Although further research is necessary to clarify pyruvate’s sole therapeutical role in critically ill patients’ immunonutrition, the first scientific successes seem to be very promising.

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

Introduction

Increasing evidence suggests that the ketone pyruvic acid (the simplest α-keto acid) may have immunonutritional significance in the modulation of PMN host defence mechanisms and granulocytic immunoregulation because of its relevant role in cellular energetics and metabolism (i.e. as an important source of respiratory cellular fuel or further metabolic precursors). Indeed, in all prokaryotic or eukaryotic cells its carboxylate anion (known as pyruvate) is certainly an important chemical compound in biochemistry and therefore it is no particular surprise that pyruvate not only can be produced by different pathways (i.e. via glycolysis in which one molecule of glucose breaks down into two molecules of pyruvate or reversible transamination reactions, etc.), but also unites several key metabolic processes (Agam and Gutman 1972; Curi et al. 1989, 1988; Fauth et al. 1993 and 1990; Frei et al. 1975; Fuchs et al. 1994; Homem de Bittencourt et al. 1993; Ing et al. 1997; Newscholme et al. 1987; Wu et al. 2005 and 2003).
In euukaryotic cells, for example, pyruvate is converted into acetyl-CoA, releasing NADH and carbon dioxide in a process called pyruvate decarboxylation (Fauth et al. 1993 and 1990; Fink 2008, 2007a, b, 2004; Willems et al. 1978). This oxidative conversion into acetyl-CoA, which is the main input for a series of reactions named after Hans Adolf Krebs (awarded the 1953 Nobel Prize) is usually catalysed by the pyruvate dehydrogenase complex as part of aerobic respiration (Krebs 1940; 1938). Interestingly, the pyruvate dehydrogenation complex as part of aerobic metabolism (Newsholme et al. 1987; Stjernholm et al. 1969; Venizelos and Hagenfeldt 1985). Additionally, when oxygen is absent or in short supply, pyruvate can also be transformed to lactate by lactate dehydrogenase (LDH) (Gardner and Leese 1988; Serrano and Curi 1989; Willems et al. 1978). LDH is an ubiquitous enzyme and therefore present in a wide variety of cells, including neutrophils. It also catalyses the concomitant interconversion of NADH and NAD⁺.

The goals of this study are therefore to document the effects of pyruvate incubated in whole blood on neutrophil-free intracellular amino acid and α-keto acid concentrations (regarding its role in PMN immunonutrition) as well as on the activities of released myeloperoxidase (MPO) and the formation of both superoxide anions (O₂⁻) and hydrogen peroxide (H₂O₂) (as markers of neutrophil function, and in order to show possible parallels with changes in neutrophil amino acid concentrations).

Materials and methods

The study was approved by the local ethics committee of the Justus Liebig University, Giessen, Germany. Ten men between 24 and 41 years (31 ± 6.3) with an average height of 179.8 cm (range 173–188) and weight of 82.6 kg (range 75–94) were selected. Those men with metabolic disease (e.g. diabetes, etc.), cardiopulmonary, neurological, allergic diseases or 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.

Pyruvate

1. To document any dose-dependent effects, whole blood samples were incubated with different pyruvate concentrations (0, 50, 100, 200 or 500 μM and 1 mM) for 120 min. The selected pyruvate concentration corresponded to 0-, ½-, 1-, 2-, 5- and 10-fold the clinically achieved plasma concentrations (see Mühling et al. (2003) and Riedel et al. (1992) for physiological values).
2. To examine whether there would be a critical duration of exposure needed to produce any significant effects, whole blood samples were incubated with pyruvate (1 mM) for 10, 60 or 120 min.

Solutions of pyruvate 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 pyruvate concentrations were as described above) at 37°C using a vibrating water bath. Corresponding volumes of HBSS were added to the control tubes. Prior to processing further, 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

PMN cell fractionation and PMN cytolysis from whole blood after incubation with pyruvate were particularly interesting aspects of this study concerning the further analysis which followed [precise details of our PMN-separation technique have been described previously (Krumholz et al. 1995, 1993; Mühling et al. 2003, 1999). Only preparation procedures, which prevent further metabolic activity and thereby reflect metabolic state at the time of sampling are appropriate for: (1) allowing intragranulocyte free amino and α-keto acid metabolism or immune functions to be analysed with a high degree of accuracy and precision, and (2) detecting small changes in metabolism. Thus, the goal must be to accomplish the fastest possible separation that retains complete activity, preserves cellular viability (without premature destruction of cellular integrity) and achieves a high degree of cell purity (Haslett et al. 1985; Rebecchi et al. 2000; Welters 2002). Moreover, continuous mechanical and chemical manipulation, changing temperatures, as well as sucrose containing density gradients, etc., may induce adverse effects on PMN due to a PMN activation such as spontaneous deformation, increased activities of extracellular released myeloperoxidase, elevated interleukin-8-transcription, increased PMN adhesion to glass surfaces, reduced chemotaxis or increased expression of complement receptors (Berger et al. 1984; Dooley et al. 1982; Welters 2002). Preliminary studies showed that despite these precautions, an activation of PMN could not always be avoided. In this study those cell suspensions identified, however, were removed from further analysis and evaluation (i.e. as indicated by a microscopic or macroscopic PMN aggregation). Our recent results, again, indicated that it was always necessary to check cell purity and viability to ensure precise and valid PML analysis. Moreover, these and former interesting findings were also an important reason for us to choose for all incubations a whole blood model (Krumholz et al. 1995, 1993; McCarthy et al. 1990; Welters 2002; Zhao et al. 2003). Another advantage is here, that many structures of an inflammatory matrix will remain during the incubation, since the immunological response of PMN mainly depends on the interactions with other blood or endothelial cells as well as on the cellular and immunological processes in the inflammatory area itself. Thus, important parameters remain stable during the incubation (i.e. continuous or physiological food and substrate supply for the PMN, maintained interactions with other blood cells, plasmatic inflammatory parameters, proteins, proteases or enzymes, etc.). For this reason there is an important advantage: the following findings in PMN are the results of nutrient interaction and biochemical communication of PMN with all structures located in whole blood and not only the results of a physiologically, biochemically and nutritionally environment-deprived single cell type. This offers a maximum proximity to the clinical question: do patients with serious diseases have a benefit from pyruvate-enriched immunonutrition?

For the above reasons, we did not choose a sucrose containing density gradient (i.e. Ficoll®) but Percoll®-gradient method for granulocyte separation. Our procedure is a further development of the methods described by Eggleton et al. (1989) and Krumholz et al. (1995, 1993), which allowed very rapid and highly selective enrichment of PMLs from very small quantities of blood. Although we used 4 ml of whole blood, 0.2 ml was enough for the isolation of sufficient granulocytes for HPLC analysis. Cooling of the whole blood samples and of the gradients to 4°C did not have an effect on granulocyte quality (i.e. as was confirmed by light microscopy), and as described above, large numbers of cells could be separated with a high level of vitality, even from small quantities of whole blood. Cell yields were determined at the same time that vitality was measured. For duplicate determination, the percentage deviation in the numbers of cells amounted to less than 2%. 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 pre-cooled (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 was 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\textsuperscript{2+}/Mg\textsuperscript{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 (in this study the average purity of the separated cells was 97.6 ± 0.8% and the average viability was 98.4 ± 0.7% because samples with a PMN purity and vitality ≤95% were discarded). On average, the cell fractionation procedure lasted 35 ± 6 min. Immediately after preparation, the extracted PMN samples were frozen at −80°C before lyophilisation (freeze dryer CIT-2®/C210, 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 analysed within a period not exceeding 4 weeks. The purity, determined in duplicate in the first aliquot by dyeing with “Türk’s Solution®” (Merck) and viability, determined in the second aliquot by exclusion of “Trypan Blue®” (Merck) was 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 <95% were discarded. In parallel, plasma samples (100 μl) were separated, lyophilised and stored using known techniques. Overall, 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.

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 method reproducibility and reproducibilities of retention times) were also within normal ranges (for details see Mühling et al. 2003, 1999). PMN amino acid concentrations are given in 10\textsuperscript{−16} mol per neutrophil-cell; PMN α-keto acid concentrations are given in 10\textsuperscript{−17} mol per neutrophil-cell.

Preparation of derivatization reagent

For the fluorescence labelling 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 oxidised reagent causes variation in the fluorescence intensity) the brown powder must be re-crystallised prior to use. Although the amount of reactive OPD is less when using the oxidised form of the reagent, this re-crystallisation 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\textsubscript{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 (σ) and 10 μl of 2-mercaptoethanol (σ) was added to yield OPD-HCl-ME. This reagent solution was stable for several hours without loss in sensitivity (Fuchs et al. 1994).

Standard samples and precolumn derivatization procedure

Analytically pure α-keto acids (σ) were dissolved in distilled H\textsubscript{2}O (Merck) containing 4% human serum albumin (Merck), immediately lyophilised and stored at −80°C. The lyophilisates (PMN, plasma and standard samples) were solubilised in 250 μl of pure methanol (Mallinckrodt Baker B.V., Deventer, Holland). The methanol also contained the α-keto acid, α-ketovalerate (KV; σ) as an HPLC internal standard. KV is a non-physiological α-keto acid. After a 3-min incubation and a 3-min centrifugation step (3000×g, Rotixa/KS®), Tuttlingen, Germany), 200 μl of the extracts was dried under N\textsubscript{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, σ) was added to the samples and mixed for 7 min in a 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 ethyl acetate portions were dried under N\textsubscript{2} (30 min), re-solubilised 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 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) chromatograms ([area_{keto acid—s}/area_{internal standard—s}] × amount or concentration of keto acids injected = calculation factor (CF); [area_{keto acid—s}/area_{internal standard—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 3-channel degasser (Knauer, Berlin, Germany) see Mühling et al. (2003, 1999).

Superoxide anion production

Superoxide anion and hydrogen peroxide production as well as activity of released myeloperoxidase were determined photometrically using modifications of known methods validated in our institute for this purpose (for further details see Krumholz et al. 1995, 1993; Mühling et al. 2007, 2006, 2005, 2004, 2002). 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 aliquoted and frozen at −20°C. Oposonized 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 centrifugation at 350×g (10 min) opsonized zymosan was re-suspended in 10 mL PBS®-glucose buffer, aliquoted and frozen at −20°C. After incubation of whole blood with pyruvate at 37°C, PMN was isolated as described above. Afterwards, the PMN was then isolated using a modification of our PMN-separation technique (as mentioned above). After stepwise (15 min and 5 min) centrifugation procedures (350×g, 20°C) as well as careful lysis of a few erythrocytes contaminating the pellet, the pelleted PMN cells were re-suspended 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 cytochrome C and 500 µL isolated PMN suspension (0.8 × 10^9/mL) and again pyruvate to be tested, were poured into a test tube. A preparation containing 500 µL buffer instead of zymosan was used as a zero adjustment. After further incubation for 15 min at 37°C the reaction was stopped by putting the test tube into ice water. After centrifugation (350×g; 5 min, 4°C) extinction of the supernatant was measured photometrically (546 nm; Digital photometer 6114S®; Eppendorf, Germany). The amount of superoxide anion produced was calculated and resulted from the extinction coefficient of cytochrome C according to the Lambert–Beer law. 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 horseradish peroxidase catalysed by 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 beenstimulated by opsonized zymosan. Phenol red was dissolved in double-distilled water (10 g/L). Horseradish peroxidase was dissolved in PBS®-glucose buffer (5 g/L). After incubation of whole blood with pyruvate at 37°C, PMN was isolated as described above. Isolated PMN was stimulated by opsonized zymosan (Sigma, Deisenhofen, Germany). The final test preparation consisted of 500 µL zymosan, 125 µL pool serum, 12.5 µL horseradish peroxidase, 12.5 µL phenol red, 12.5 µL sodium azide (200 mmol/L/l; Merck, Darmstadt, Germany), 500 µL PMN suspension (2 × 10^6 PMN-cells/mL) and again pyruvate. 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 were prepared, incubated and measured identically.

Activity of released myeloperoxidase

Activity of released myeloperoxidase was also determined photometrically. 1 mmol/L 2,2′-azino-di-(3-ethylbenz-thiazoline) 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 pyruvate 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 pyruvate (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 x 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 cooperation with colleagues from the Department of Medical Statistics, Justus Liebig University, Giessen, Germany. 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 ≤ 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 ≤ 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 χ-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. 2003, 1999) (Table 1). Intracellular pyruvate levels amounted on average to 6.36 × 10^{-17} mol per neutrophil (PMN) cell (≈ 158 μmol/L PMN cell volume [NCV]; plasma: 117.6 ± 42 μmol/L). The intracellular χ-ketoglutarate content at almost 1.28 × 10^{-17} mol per PMN cell (≈ 32 μmol/L NCV; plasma: 7.6 ± 2.1 μmol/L) was approximately 3.7 times lower. The intragranulocytic amino acids glutamine, glutamate and alanine associated with pyruvate metabolism could also be exactly quantified: their average concentrations at 3.11 ± 10^{-16} mol per PMN cell (glutamine) [≈ 773 μmol/L NCV; plasma: 546 ± 191 μmol/L], 5.91 ± 10^{-16} mol per PMN cell (glutamate) [≈ 1.470 μmol/L NCV; plasma: 31 ± 7 μmol/L], and 1.94 × 10^{-16} mol per PMN cell (alanine) [≈ 482 μmol/L NCV; plasma: 354 ± 83 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) [≈ 97 μmol/L NCV; plasma: 61 ± 19 μmol/L], 2.65 × 10^{-16} mol (aspartate) [≈ 659 μmol/L NCV; plasma: 13 ± 4 μmol/L]), 0.35 × 10^{-16} mol (arginine)...

Table 1: Effects of pyruvate (PYR; 1 mM) incubated with whole blood for 10, 60 and 120 min on important free intracellular amino acids (10^{-16} Mol per PMN-cell; mean ± SD; n = 10). χ-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 Mean | 10 min SD | 60 min Mean | 60 min SD | 120 min Mean | 120 min SD |
|----------|-------------|-----------|-------------|-----------|--------------|-----------|
| Asparagine | 0.39 | 0.10 | 0.41 | 0.09 | 0.37 | 0.09 |
| Glutamine  | 3.11 | 0.77 | 3.32 | 0.91 | 2.90 | 0.86 |
| Aspartate  | 2.65 | 0.73 | 2.80 | 0.61 | 2.56 | 0.57 |
| Glutamate  | 5.91 | 1.16 | 6.28 | 1.81 | 5.79 | 1.56 |
| Ornithine  | 0.46 | 0.08 | 0.49 | 0.08 | 0.45 | 0.09 |
| Arginine   | 0.35 | 0.07 | 0.37 | 0.07 | 0.34 | 0.08 |
| Serine     | 2.30 | 0.44 | 2.22 | 0.53 | 2.07 | 0.45 |
| Glycine    | 2.47 | 0.56 | 2.35 | 0.49 | 2.29 | 0.58 |
| Alanine    | 1.94 | 0.46 | 1.86 | 0.46 | 1.81 | 0.59 |
| χ-Ketoglutarate | 1.28 | 0.30 | 1.25 | 0.34 | 1.17 | 0.35 |
| PYRUVATE   | 6.36 | 1.84 | 6.14 | 1.93 | 5.92 | 1.56 |
| O_2^-      | 3.608 | 0.959 | 3.484 | 0.886 | 3.264 | 0.773 |
| H_2O_2     | 1.279 | 0.316 | 1.263 | 0.365 | 1.198 | 0.304 |
| MPO        | 0.584 | 0.157 | 0.545 | 0.190 | 0.499 | 0.172 |

PYR (1 mM)

Asparagine | 0.40 | 0.11 | 0.53 | a | 0.12 | 0.65 | a |
| Glutamine  | 2.92 | 0.78 | 4.62 | a | 1.27 | 5.54 | a |
| Aspartate  | 2.87 | 0.80 | 4.27 | a | 1.32 | 5.18 | a |
| Glutamate  | 6.90 | 1.45 | 11.66 | a | 3.91 | 13.84 | a |
| Ornithine  | 0.48 | 0.11 | 0.58 | a | 0.14 | 0.73 | a |
| Arginine   | 0.36 | 0.10 | 0.42 | a | 0.11 | 0.48 | a |
| Citrulline | 0.13 | 0.03 | 0.14 | a | 0.04 | 0.16 | a |
| Serine     | 2.19 | 0.46 | 2.52 | a | 0.67 | 3.01 | a |
| Glycine    | 2.22 | 0.44 | 2.54 | a | 0.59 | 3.41 | a |
| Alanine    | 2.26 | 0.57 | 3.34 | a | 0.98 | 5.05 | a |
| χ-Ketoglutarate | 1.34 | 0.37 | 2.01 | a | 0.57 | 2.78 | a |
| PYRUVATE   | 7.23 | 2.29 | 12.07 | a | 3.36 | 19.31 | a |
| O_2^-      | 4.652 | 1.195 | 10.306 | a | 3.051 | 17.091 | a |
| H_2O_2     | 1.565 | 0.437 | 3.182 | a | 0.846 | 4.992 | a |
| MPO        | 0.672 | 0.211 | 1.208 | a | 0.413 | 1.502 | a |

* p ≤ 0.05 versus control values; # p ≤ 0.05 versus 10 min
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[≈ 87 μmol/L NCV; plasma: 79 ± 20 μmol/L], 0.46 × 10^{-16} mol (ornithine) [≈ 114 μmol/L NCV; plasma: 42 ± 20 μmol/L], 2.30 × 10^{-16} mol (serine) [≈ 572 μmol/L NCV; plasma: 118 ± 33 μmol/L], 2.47 × 10^{-16} mol (glycine) [≈ 614 μmol/L NCV; plasma: 251 ± 53 μmol/L]) and 0.14 × 10^{-16} mol (citrulline) [≈ 34.8 μmol/L NCV; plasma: 32 ± 9 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: 1.3), glutamine (i.e: 1.4), alanine (i.e: 1.3), arginine (i.e: 1.1), serine (i.e: 4.8) and glycine (i.e: 2.4) not only high plasma but also high intracellular concentrations have been found, while α-ketoglutarate (i.e: 4.2), glutamate (i.e: 47.4), aspartate (i.e: 50.6), asparagine (i.e: 1.6), ornithine (i.e: 2.7) with low plasma concentrations apparently accumulated within the neutrophils.

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

1 mM pyruvate significantly increased pyruvate and α-ketoglutarate profiles in a duration of exposure dependent manner (PMN incubation for ≥60 min, Table 1). Following low pyruvate doses (50 μM, PMN incubation for 120 min), concentrations of free intracellular α-keto acids remained unaffected. In the presence of higher pyruvate concentrations (PMN incubation for 120 min) significant dose-dependent increases in PMN pyruvate and α-ketoglutarate (≥100 μM) were observed (Fig. 1). PMN free α-ketobutyrate, α-ketoisovalerate, α-ketoisocapronate, p-hydroxy-phenylpyruvate and α-keto-β-methyl-valerate profiles remained unaffected.

Effects of pyruvate on free amino acid pool in PMN

Concentrations of free intracellular amino acids were unaffected by 50 μM pyruvate (PMN incubation for 120 min), respectively. Following higher pyruvate concentrations (PMN incubation for 120 min), significant dose-dependent increases in PMN glutamate, asparagine, glutamine, aspartate, alanine (≥100 μM, Fig. 2), ornithine and arginine (≥500 μM, Fig. 3), as well as in serine and glycine (≥1 mM, Fig. 4) were observed. Moreover, 1 mM pyruvate (PMN incubation for ≥60 min) significantly increased PMN glutamate, glutamine, asparagine, aspartate, alanine, ornithine, arginine, glycine and serine in duration of exposure-dependent manner (Table 1). PMN-free citrulline, lysine, leucine, valine, methionine, taurine, hypotaurine, threonine, a-aminobutyrate, tyrosine, tryptophane, phenylalanine and histidine profiles remained unaffected.

Effects of pyruvate on oxidative response and myeloperoxidase activity

All PMN immune functions tested were unaffected up to 50 μM pyruvate. In the presence of higher pyruvate concentrations (PMN incubation for 120 min) significant dose-dependent increases in PMN pyruvate and α-ketoglutarate (≥100 μM) were observed (Fig. 1).

**Fig. 1** Free intracellular pyruvate (PYR) and α-ketoglutarate (KG) concentrations in PMN-cells following pyruvate incubation (0, 50, 100, 200, 500 μM and 1 mM; 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 ≤ 0.05 versus control values

**Fig. 2** Free intracellular glutamine (gln), glutamate (glu), asparagine (asn), aspartate (asp), and alanine (ala) concentrations in PMN-cells following pyruvate incubation (0, 50, 100, 200, 500 μM and 1 mM; 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 ≤ 0.05 versus control values
supplementation superoxide anion generation (≥100 μM), hydrogen peroxide formation (≥100 μM) and MPO activity (≥200 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**

For the first time the immunonutritional role of pyruvate on neutrophils (PMN), free α-keto and amino acid profiles, important reactive oxygen species (ROS) produced [superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂)] as well as released myeloperoxidase (MPO) activity has been investigated. The results of our study presented here showed that exogenous pyruvate—incubated in whole blood—led to a significant increase in PMN intracellular pyruvate content, indicating a relevant transport across the plasmatic cell membrane. Indeed, essential to the roles of monocarboxylates such as lactate and pyruvate in cellular metabolism and metabolic communication between tissues is their rapid transport through different cell membranes, which for example is catalysed by a recently identified family of proton-linked monocarboxylate transporters (MCT’s) or simply by diffusion (Brivet et al. 2003; Burckhardt and Burckhardt 2003; Kielucka et al. 1981; Kozlova et al. 1983; Lamers and Hulsmann 1975). MCT’s are located at the plasma but also subcellular membranes (i.e. mitochondria) of human cells and classically transport metabolites across plasma membranes, directionally controlled by proton and metabolic concentrations and independently of energy input (Hume et al. 1978; Jolkver et al. 2009; Merezhinskaya and Fishbein 2009). Moreover, there is scientific evidence for the existence of a facilitated diffusion carrier, which specifically mediates the translocation
of pyruvate. This transport system, which has been found in the plasma membrane of Trypanosoma brucei (bloodstream form), differs from other known monocarboxylate carriers present in the mitochondrial and/or plasma membrane of eukaryotic cells because of its high specificity for pyruvate and due to the fact that it does not transport \( L-lactate \) (Edlund and Halestrap 1988; Halestrap 1978; Poole et al. 1989; Wiemer et al. 1995). Interestingly, some important drugs may influence pyruvate transport mechanisms or even cellular pyruvate degradation. For example, Aires et al. (2008) reported the inhibition of the mitochondrial pyruvate uptake by the anticonvulsive drug valproic acid. But different antipsychotic drugs with clinically equivalent doses (i.e. chlorpromazine, thioridazine, thiothixene, etc.) are able to inhibit the pyruvate dehydrogenase complex (PDHC) to varying extents, too (Bakowski and Parekh 2007; Chen et al. 1998; Zhang et al. 2009).

Furthermore, although our study does not allow to infer whether the effects were direct (i.e. following uptake of important substrates by neutrophils produced from other blood cells) or metabolically induced changes brought about by pyruvate incubation of whole blood (i.e. following intracellular pyruvate conversion), however, referring to our findings we also note an relevant role of pyruvate as an important intragranulocyte substrate of various \( z \)-keto and amino acid pathways (Agam and Gutman 1972; Mahiout et al. 1997; Miwa et al. 2000; Newsholme et al. 1987; Selvaraj and Sbarra 1966; Serrano and Curi 1989; Vaartjes et al. 1979; Willems et al. 1978). Since intracellular pyruvate increases the concentrations of \( z \)-ketoglutarate 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. A more precise look at the constitution of PMN amino and \( z \)-keto acid changes favour the hypothesis that increases in neutrophil free pyruvate concentrations are mainly followed by pyruvate conversion into important amino and \( z \)-keto acid derivatives (Curi et al. 1989, 1988; Fuchs et al. 1994; Mühling et al. 2010, 2007, 2005, 2002). Indeed, our assumption that this metabolisation processes also occurs within PMN cells, may particularly be supported with regard to very similar immunonutritional findings induced by \( z \)-keto glutarate, alanyl-glutamine, ornithine or arginine in other leukocyte cells or even neutrophils. We therefore strongly suggest that pyruvate may act as a preliminary stage substrate for subsequent intracellular metabolization. Interestingly, one of the most important cellular biochemical processes in which pyruvate is involved is its oxidative degradation, catalyzed by the pyruvate dehydrogenase complex (PDH) as part of aerobic respiration. The conversion—very far from equilibrium—of pyruvate to form acetyl-CoA-accessorily releasing NADH and CO_{2} via decarboxylation is known as an important link reaction between the metabolic pathways of glycolysis and of course the citric acid cycle. There are either used as a substrate for oxidative phosphorylation, therefore serving as a respiratory fuel source or as a precursor of many synthetic intermediates and metabolic pathways (i.e. urea cycle [i.e. in the form of aspartate produced from oxalacetate] fatty acid, isoprenoid biosynthesis, etc.) (Agam and Gutman 1972; Board and Newsholme 1996, 1990; Brown et al. 2004; Newsholme et al. 1987; Stjernholm et al. 1969; Witko-Sarsat et al. 2000). Indeed, an absence of this enzyme complex has serious implications for the affected patients. For example, malfunction of the citric acid cycle due to PDH deficiency deprives the body of energy and leads to an abnormal buildup of lactate resulting in lactic acidosis in newborns and often presents with clinical signs like severe lethargy, poor feeding, tachypnea or cases of death (Curi et al. 1989 and 1988; Gardner and Leese 1988; Paul et al. 1987; Su et al. 2007; Venizelos and Hagenfeldt 1985). But for neutrophils, especially, the reversible transamination of pyruvate by the alanine aminotransferase (also known as glutamic pyruvic transaminase, GPT), also seems to be of further importance concerning their physiological tasks in whole body immune defense. In this case amino groups are transferred from glutamate to pyruvate producing alanine and \( z \)-ketoglutarate which themselves in turn can directly be shunted into the tricarboxylic acid cycle and oxidatively decarboxylated to succinyl-CoA by the \( z \)-ketoglutarate dehydrogenase complex (Fink 2004; Frei et al. 1975; Taylor et al. 2005; Willems et al. 1978). Furthermore, pyruvate may also be catalysed by an irreversible anaplerotic carboxylation (metabolized by pyruvate carboxylase), which provides oxalacetate precursors for the citric acid cycle but also—after conversion into phosphoenolpyruvate—for gluconeogenesis. Evidence for this exists as key enzymes for gluconeogenesis such as fructose-1,6-bisphosphatase had also been found in leukocytes (Agam and Gutman 1972; Fauth et al. 1993, 1990; Fink 2008 and 2004; Frei et al. 1975; Mackenzie and Lever 2007; Stjernhome et al. 1969). Moreover, pyruvate may also be a starting point for the “de novo synthesis” of alanine, serine and glycine as well as glucose. Enzymes required for this, especially NADPH-forming metabolic pathways, are certainly present in PMN cells as others have shown (Agam and Gutman 1972; Curi et al. 1989 and 1988; Fink 2008; Frei et al. 1975; Fuchs et al. 1994; Engel et al. 2009a, b; Mühling et al. 2010 and 2005; Newsholme et al. 1987; Serrano and Curi 1989). Concerning to the present but also former findings we therefore strongly believe, that the indirect availability of pyruvate in the form of glutamate, glutamine, \( z \)-ketoglutarate, alanine, asparagine, aspartate, arginine, ornithine, serine or glycine...
may emphasise the fundamental importance of this molecule in restoring pathophysiological depleted amino and α-keto acid pools (Mühleng et al. 2001). Thus indirect regulation of arginine, ornithine or aspartate (i.e. via oxalacetate) metabolism (for example used as a substrate for the enzymes of the urea cycle or formation of phosphoserine and serine), the synthesis of •NO required for PMN activation and ultimately the formation of glutamine, glutamate or α-ketoglutarate metabolism (i.e. used for glutathione, proline, purine and pyrimidine synthesis, export of glutamate in exchange for import of cystine and following conversion to cysteine, synthesis of glutathione, proline, purine and pyrimidine synthesis, glutamate or 1972; Curi et al. 1989, 1988; Fink 2008; Frei et al. 1975; Fuchs et al. 1994; Engel et al. 2009a, b; Mühleng et al. 2010, 2005; Newsholme et al. 1987; Serrano and Curi 1989; Wu et al. 2005, 2003).

Nevertheless, the conversion of pyruvate does not just supply important precursors for the above-mentioned metabolic pathways, since it also provides energy-rich molecules such as nicotinamide adenine dinucleotide phosphate (NADPH) or guanosine-5′-triphosphate (GTP). For example NADPH can not only arise from the conversion of pyruvate by pyruvate dehydrogenase, but also from the catalytic activities of the TCA cycle enzymes isocitrate, α-ketoglutarate and malate dehydrogenase (Board et al. 1990; Brown et al. 2004; Chang et al. 1977; Mühleng et al. 2010). Moreover, succinyl-CoA synthetase catalyzes another important step in the TCA cycle which involves the substrate-level phosphorylation of guanosine-5′-diphosphate to GTP, which acts as a important source of energy or as an activator of substrates in metabolic reactions (i.e. protein synthesis), like that of ATP, but more specific. Indeed, Hyslop et al. (1984) found that neutrophil homogenates contained a high affinity guanosine triphosphatase (GTPase) activity during chemotactic peptide stimulation in neutrophils. Furthermore, Woodman et al. (1988) described a relevant role for de novo protein synthesis in maintaining or augmentation of the chemotactic peptide-induced respiratory burst in neutrophils, manufacturing O2−. However, a closer look to granulocytic immune functions shows, that not only GTPase but also NADPH-oxidase (NADPH-O) in particular, which can be found in the plasma membrane as well as in the membrane of phagosomes, plays a pivotal role in human immune regulation (Kang et al. 2002; Klein et al. 1990; Kobayashi et al. 2001). The complex is normally latent in neutrophils and is activated to assemble in the membranes during respiratory burst (Mühleng et al. 2010, 2006, 2005, 2004, 2002). It primarily generates superoxide, a highly reactive free radical, by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen. In phagosomes or outside the cell, superoxide can spontaneously form hydrogen peroxide that will undergo further reactions to generate reactive oxygen species (ROS) (Newsholme et al. 1987; Wu et al. 2005, 2003; Zhou and Yu 1998). Superoxide is capable of killing bacteria and fungi by mechanisms that are not yet fully understood. It also may inactivate critical metabolic enzymes, initiate lipid peroxidation and liberate redox active iron, which allows the generation of indiscriminate oxidants such as the hydroxyl radical. Superoxide probably kills bacteria directly as the virulence of many pathogens is dramatically attenuated when their superoxide dismutase (SOD) genes are deleted (Engel et al. 2009a, b; Mühleng et al. 2010, 2006, 2005, 2004, 2002; Wu et al. 2005, 2003). Therefore, superoxide is one of the main reactive oxygen species in neutrophils and as such, SOD plays a key antioxidant role. However, downstream products of superoxide also include hydrogen peroxide (H2O2) and hypochlorous acid. In activated neutrophils during the respiratory burst, HOCl is produced by myeloperoxidase-mediated (MPO) peroxidation of chloride ions from H2O2. Indeed, a number of authors described that HOCl contributes to the destruction of bacteria by inhibition of important biochemical pathways (i.e. glucose oxidation (Knox et al. 1948)) or reacting with a wide variety of their biomolecules including depletion of adenine nucleotides (i.e. ATP hydrolysis), DNA and RNA (i.e. inhibition of replication) fatty acid groups (i.e. lipid hydrolysis which affect membrane permeability), cholesterol (forming toxic chlorhydrins) as well as post-translational modifications to proteins (i.e. protein unfolding and aggregation) (i.e. Cicaless et al. 1996a, 1996b; Dahlgren and Karlsson 1999; Ing et al. 1997, Huang et al. 2009, Wu et al. 2005, 2003). But regarding the “positive” or “desired” bacteriocidal nature of chlorine solutions induced by HOCl one should bear in mind that the diapedesis of immunonutritionally (i.e. following pyruvate) improved neutrophils in inflammationally damaged extravascular matrices may also paradoxically wreak serious additional granulocyte-mediated injury to all cells or tissue structures with the potential risks of aggravating the course of the disease accidentally. Interestingly, recent findings showed that pyruvate may also have some antioxidant and anti-inflammatory actions as an effective scavenger of highly reactive oxygen species, for example produced by neutrophils (Engel et al. 2009a, 2009b; Mate’s et al. 2008; Mühleng et al. 2010, 2006, 2005, 2004, 2002). Overall, referring to our results as well as regarding the beneficial immunonutritional effects found by others, it seems clear that pyruvate 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. Unfortunately, any further clarification of pyruvate’s sole role of
in vivo therapeutic immunonutritional properties may be associated with important galenical problems, mainly due to its instability in aqueous solutions. For this reason, current results which investigated ethyl pyruvate (EP), a simple aliphatic ester derived from pyruvate, seems very promising (Fink 2004; Zingarelli 2004). EP has shown to be safe at clinically relevant doses treatment with EP has been shown to improve survival and/or ameliorating organ dysfunction in a wide variety of clinical models of critical illness as well as it has beneficial effects concerning disturbed cellular function in vitro (Cai et al. 2009; Cheng et al. 2007; Fink 2007a, b; Genovese et al. 2009; Lee et al. 2008; Tsung et al. 2005; Wang et al. 2009; Yang et al. 2009; Zhang et al. 2009). For example, EP has demonstrated anti-inflammatory actions, improved bacterial translocation or hyperpermeability due to endotoxinemia and may also has benefit in models of sepsis and septic shock by inhibiting tumor necrosis factor-alpha production and interleukin-6 mRNA expression in several tissues, or through a reduction of the enzyme activities of cyclooxygenase-2 or inducible nitric oxide synthase (Dave et al. 2009; Deng et al. 2009; Di et al. 2009; Hollenbach et al. 2008; Liang et al. 2009; Su et al. 2007; Taylor et al. 2005; van Zoelen et al. 2006). But the beneficial anti-inflammatory effects may also be due to modifications in the circulating levels of high mobility group box-1 and NF-kappaB signalling pathways (Fink 2008; Han et al. 2005; Matsumoto et al. 1991; Johansson et al. 2008; Su et al. 2008; Varma et al. 2006).

Although further research is necessary to clarify pyruvate’s sole therapeutical role (for example given as EP) in critically ill patients immunonutrition, the first scientific successes seem to be very promising.

Conflict of interest The authors have no relationship, financial or otherwise, with individuals or organizations that could influence the author’s work inappropriately, and a conflict of interest does not exist.

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