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The effects of thiopentone on free intracellular amino acids in polymorphonuclear leucocytes

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Summary. Previous studies have shown the inhibitory effects of thiopentone on polymorphonuclear leucocyte (PML) function. However, major biochemical mechanisms which have been involved are still unknown. The aim of this study was therefore to investigate thiopentone's effects on intracellular amino acid metabolism in PML using both advanced PML separation – and HPLC techniques, especially developed for this purpose and precisely validated in our institute. Overall, our study indicates important dose-dependent alterations of free intracellular amino acid metabolism following thiopentone treatment and draw attention to the biochemical mechanisms which may be involved in both thiopentone-induced modulation in PML function and cellular immunocompetence.

Keywords: Amino acids – Leucocytes – Thiopentone – Intracellular amino acids – High-performance liquid chromatography – Amino acid metabolism

Introduction

Polymorphonuclear leucocytes (PML) are playing crucial role in the human host-defense system which protects human organism from pathogenic germs. Derangement of any PML function (i.e. following treatment with hypnotic agents) may allow bacterial infection to develop and might contribute to mortality and morbidity (Al-Sawaf et al., 1993). These anesthetics may not lead to perioperative immunosuppresion (i.e. phagocytosis, adherence, chemotaxis etc.) but may potentially further compromise an already depressed host-defense mechanism if used to sedate critically ill patients. Thiopentone is known to impair cellular functions of immunocompetent cells, including PML, both *in vitro* and *in vivo* (Heller et al., 1998; Nishina et al., 1998; Krumholz et al., 1995). Until now, however, there are only few findings regarding the influence of barbiturates on the biochemical mechanisms which

may be involved with alterations in cell function (Amakawa et al., 1996; Goodman et al., 1996). No reports exist especially regarding free intracellular amino acid metabolism of major immunocompetent cells following thiopentone treatment. Thiopentone is a short acting barbiturate which plays an important part in modern anesthesia and is frequently used. Therefore its influence on major biochemical pathways in PML is of important interest.

The goal of this study was therefore to quantify the effects of different thiopentone concentrations on free intracellular amino acid metabolism in living polymorphonuclear leucocytes – as valuable tools for evaluating amino acid metabolism in nucleated cells – using novel, highly-advanced, PML separation and high-performance liquid chromatography techniques; recently developed and precisly validated in our institute (Mühling et al., 1999).

Material and methods

The study was approved by the local ethics committee of the Justus-Liebig-University, Giessen. Thirty-two male volunteers between 25 and 36 years (mean 29 years) with an average height of 179 cm (range 174–192) and weight of 79 kg (range 72–96) were selected: those volunteers with metabolic (diabetes etc.), cardiopulmonary, neurological or allergic diseases as well as those taking any drugs were exluded from the study. 40 ml whole blood samples (drawn into heparinized plastic tubes) were taken from each volunteer between 09:00 and 09:30 a.m. to exclude potential circadian variations.

Thiopentone incubation

Subsequently 10 ml of whole blood each was incubated either with 0 μg/ml (Control), 5 μg/ml, 20 μg/ml or 200 μg/ml thiopentone for 1 hour (37°C, water bath). The concentrations of thiopentone correspond at both clinical concentrations and at 10 times this concentration (Doenicke, 1965). Solutions of thiopentone were prepared in Hank's balanced salt solution (HBSS) and the pH confirmed to be 7.4. The 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 phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin, as well as 10 μg/ml antipain (all acquired from Sigma, USA) were added to each plastic heparin tube before the blood samples; these additions served to inhibit proteases.

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

Precise details our novel PML-separation techniques have previously been described (Mühling et al., 1999). Extraction of PML was accomplished using a cooled (4°C) Percoll®-gradient. The cooled and heparinized whole blood samples were then overlaid into a previously prepared and precooled (4°C) Percoll®-gradient before centrifugation. After this cellular separation the PML were carefully taken from the sample and suspended in cooled (4°C) and diluted PBS® stock buffer. After a second centrifugation, the PBS® stock buffer was discarded and the erythrocytes remaining in the sample were hypotonically lysed using cooled (4°C), destilled water. After 20 s the PML samples were immediately brought back to isotonicity and resuspended by adding diluted PBS® stock buffer. After a third centrifugation the PBS® stock buffer was again discarded and 2 aliquots of resuspended sample were removed. Immediately after withdrawal and preparation, the extracted and cooled PML samples were deep frozen at −80°C before

lyophilization. In aliquots of the PML samples both the purity as well as their vitality were subsequently verified by light microscopy. Samples with a PML purity <96% and those with more than 4% avitality were discarded.

Sample preparation

The lyophilizates were solubilized in an 80% methanol/20% H_2O so as to guarantee short term stability before the column derivitization procedure ("chemical preservation", see Mühling et al., 1999).

Automated precolumn derivitization

Methanolic extracts of the various lyophilized samples were prepared as previously described (Mühling et al., 1999). After incubation and centrifugation procedures samples were transferred to a special sample tube where alkaline 0.5 M borate buffer and o-phthaldialdehyde-2-mercaptoethanol were automatically added. This base derivitization was stopped after exactly 120 s by neutralization with 0.75 M HCL. The mixture was then transferred to a rarefaction vial, diluted and 25 μl of this mixture was injected into the columns.

Matrix dependent calibration

Standard samples (including all amino acids which we measured in PML) have been prepared in our laboratory using a method previously described (Mühling et al., 1999). Our method has been conscentiously validated.

Equipment

The high-performance liquid chromatography system consisted of a hydrostatic gradient pump, a controller for gradient programming (600 E®, Waters, U.S.A.) and a programmable autosampler for the automated derivatization procedures (Triathlon®, Spark, The Netherlands) within a rheodyne injection valve and a 100 μl filling loop (AS 300®, Sunchrom, Germany). The following column was used for separation: Nova-Pak®, 300 mm × 3,9 mm I.D.; RP-C-18, 60 Å, 4 μm (Waters, U.S.A.). Column-temperatures were maintained at 35°C using a column oven (Knauer, Germany). Fluorescence was routinely monitored using a fluorescence spectromonitor (RF-530®, Shimadzu, Japan). Measurements were made at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. Data recording and evaluation was performed using computer integration software (EuroChrom 2000® Knauer, Germany).

Gradient

The program and the automatically degassed (3-channel degasser, Knauer, Berlin) solvents used were as previously described (Mühling et al., 1999).

Statistical analysis

After the results were demonstrated to be normally distributed (Pearson Stephens test), statistical methods included Bartlett test to check homogeneity of variance ($p \leq 0{,}1$). If the requirements were met, analysis of variance for repeated measures (ANOVA) was

Table 1. Free acid amino acid and acid amid concentrations in PML-cells from 32 healthy subjects. All probes were incubated for 1 hour. Concentrations are given in picoMol per PML-cell (Mean ± SD); *Abb.* Abbreviations, * = $p \leq 0.05$

Acid amino acids, Acid amides Abb.	Thiopentone incubation			
	Control	5 μg/ml	20 μg/ml	200 μg/ml
asparagine (asn)	0.38 ± 0.05	0.39 ± 0.06	0.36 ± 0.07	0.41 ± 0.08
glutamine (gln)	2.47 ± 0.50	2.67 ± 0.56	2.84 ± 0.68	3.18 ± 0.72*
aspartate (asp)	2.07 ± 0.26	2.04 ± 0.31	2.25 ± 0.43	2.99 ± 0.59*
glutamate (glu)	5.86 ± 0.73	5.52 ± 0.61	4.92 ± 0.61*	4.33 ± 0.51*

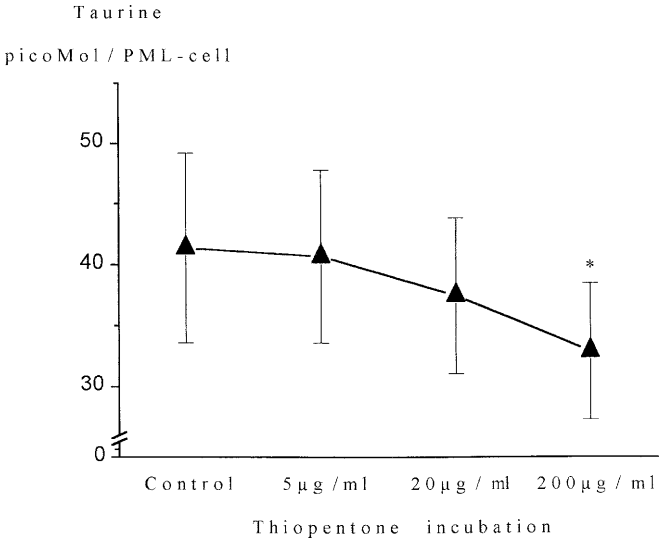

Fig. 1. Free taurine concentrations in PML-cells from 32 healthy subjects. All probes were incubated for 1 hour. Concentrations are given in picoMol per PML-cell (Mean ± SD); * = $p \leq 0.05$

appropriate. If the requirements were not fulfilled, Friedmann analysis of variance was performed. Probability levels of $p \leq 0.05$ were considered to be significant. The data are given as means ± standard deviations (mean ± SD).

Results

The amino acid concentrations obtained in the control cells showed on data beyond physiological ranges (see Mühling et al., 1999). Incubation with 5 μg/ml thiopentone caused no alterations in intracellular amino acid profiles. Incubation with 20 μg/ml thiopentone caused an increase in lysine (+15%), serine (+20%) and threonine (+23%) as well as a decrease in glutamate (−16%) concentrations. The higher concentrations of thiopentone (200 μg/ml) significantly decreased taurine (−21%), glutamate (−26%) and α-aminobutyrate (−24%) concentrations. Furthermore, incubation with 200 μg/ml thiopentone increased concentrations of glutamine (+28%), basic amino

Table 2. Free basic amino acid concentrations in PML-cells from 32 healthy subjects. All probes were incubated for 1 hour. Concentrations are given in picoMol per PML-cell (Mean ± SD); *Abb*. Abbreviations, * = $p \leq 0.05$

Basic amino acids Abb.	Thiopentone incubation			
	Control	5 μg/ml	20 μg/ml	200 μg/ml
ornithine (orn)	0.39 ± 0.11	0.40 ± 0.11	0.46 ± 0.12	0.52 ± 0.14*
lysine (lys)	0.57 ± 0.10	0.60 ± 0.11	0.67 ± 0.14*	0.73 ± 0.16*
arginine (arg)	0.27 ± 0.05	0.29 ± 0.06	0.30 ± 0.05	0.28 ± 0.05
citrulline (cit)	0.10 ± 0.02	0.10 ± 0.03	0.11 ± 0.02	0.10 ± 0.02

Table 3. Free branched chain amino acid (*BCAA*) and methionine concentrations in PML-cells from 32 healthy subjects. All probes were incubated for 1 hour. Concentrations are given in picoMol per PML-cell (Mean ± SD); *Abb*. Abbreviations, * = $p \leq 0.05$

BCAA Abb.	Thiopentone incubation			
	Control	5 μg/ml	20 μg/ml	200 μg/ml
isoleucine (ile)	0.28 ± 0.05	0.29 ± 0.05	0.32 ± 0.06	0.36 ± 0.06*
leucine (leu)	0.61 ± 0.13	0.56 ± 0.11	0.69 ± 0.14	0.77 ± 0.16*
valine (val)	0.39 ± 0.06	0.38 ± 0.06	0.42 ± 0.06	0.47 ± 0.07*
methionine (met)	0.16 ± 0.03	0.15 ± 0.03	0.16 ± 0.03	0.20 ± 0.04*

Table 4. Free neutral amino acid concentrations in PML-cells from 32 healthy subjects. All probes were incubated for 1 hour. Concentrations are given in picoMol per PML-cell (Mean ± SD); *Abb*. Abbreviations, * = $p \leq 0.05$

Neutral amino acids Abb.	Thiopentone incubation			
	Control	5 μg/ml	20 μg/ml	200 μg/ml
serine (ser)	1.51 ± 0.26	1.58 ± 0.28	1.81 ± 0.26*	1.89 ± 0.27*
glycine (gly)	2.39 ± 0.37	2.45 ± 0.38	2.67 ± 0.37	3.13 ± 0.41*
threonine (thr)	0.65 ± 0.11	0.69 ± 0.10	0.80 ± 0.13*	0.86 ± 0.12*
alanine (ala)	1.78 ± 0.31	1.69 ± 0.39	1.82 ± 0.46	2.05 ± 0.54*
α-aminobutyrate (aba)	0.21 ± 0.04	0.22 ± 0.04	0.18 ± 0.03	0.16 ± 0.03*

Table 5. Free aromatic amino acid concentrations in PML-cells from 32 healthy subjects. All probes were incubated for 1 hour. Concentrations are given in picoMol per PML-cell (Mean ± SD); *Abb*. Abbreviations, * = $p \leq 0.05$

Aromatic amino acids Abb.	Thiopentone incubation			
	Control	5 μg/ml	20 μg/ml	200 μg/ml
tyrosine (tyr)	0.29 ± 0.06	0.30 ± 0.07	0.32 ± 0.07	0.28 ± 0.08
tryptophane (trp)	0.12 ± 0.02	0.11 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
phenylalanine (phe)	0.81 ± 0.12	0.77 ± 0.14	0.83 ± 0.17	0.78 ± 0.17
histidine (his)	0.69 ± 0.17	0.66 ± 0.18	0.79 ± 0.22	0.89 ± 0.25*

acids [ornithine (+33%), lysine (+26%)], neutral amino acids [serine (+25%), glycine (+31%)], threonine (+32%), alanine (+15%) methionine (+25%) and branched chain amino acids [isoleucine (29%), leucine (+26%), valine (+21%)] significantly. The alterations of free intracellular amino acids in PMN following incubation with various thiopentone concentrations are given in Table 1 to 5 and Fig. 1. Sampling of the sober volunteers (n = 32) was performed on average at 8.21 a.m. (±11 min). The average number of PML cells that could be separated from each whole blood sample was $2.42 \times 10^6 \pm 0{,}78 \times 10^6$. The average purity of the separated cells was 98.9% ± 0.9%, while their average vitality was 99.2% ± 0.5%. Cell yields were determined at the same time that vitality was measured. Percentage deviations upon duplicate estimations of cell numbers amounted to less than 2%. The coefficients of variations for method reproducibility (coefficients of "within-day" and "between-day" variations of areas) for 10 ("within-day") and 10 ("between-day") runs of an OPA-derivatizised PML sample were between 0.89% (for methionine) and 2.35% (for ornithine) respectively between 1,73% (glutamate) and 4,56% (lysine). Reproducibilities of the retention times (coefficients of "within-day" and "between-day" variations of retention times) were between 0.02% (lysine) and 0.23% (citrulline) respectively between 0.68% (threonine) and 0.04% (lysine).

Discussion

For the first time the effects of thiopentone on free intracellular amino acid metabolism in PML has been investigated. Overall, our study indicates dose-dependent alterations in free intracellular amino acids which point to alterations in fundamental biochemical mechanisms following thiopentone treatment.

Thiopentone – a short acting barbiturate – plays an important part in modern anaesthesia and is frequently used. For this reason, concerning the influence of thiopentone on free intracellular amino acids in general, a discussion on important mechanisms by which thiopentone might act is useful. Thiopentone seems to protect neuronal damage in ischemia. Amakawa et al. (1996) found a suppression of important excitatory amino acid release of pyramidal cells in gerbins. Moreover, thiopentone appears to influence specifically and differentially important ion channel gating systems (Todorovic and Lingle, 1998; Heath and Terrar, 1997). These particular effects suggest that alterations in intracellular ions, intracellular pH and osmolarity might occur, too. Indeed, barbiturates are able to change intracellular pH as well as osmolarity via their effects on various channel currents (particularly regarding Ca^{2+} availability and Na^+/H^+-exchange). Kanaya et al. (1998) observed that thiopentone increased steady state intracellular pH and led to intracellular alkanization in ventricular myocytes which could be completely blocked by inhibition of Na^+/H^+ exchange. Not only changes in intracellular buffering but also alterations in physiological respiratory burst pathway following thiopentone may influence intracellular pH, too. Segal et al. (1981) described that impaired leucocyte microbicidal activity alters non-

mitochondrial respiration and influences intracellular pH and osmolarity. The means by which changes in intracellular pH as well as amino acid transport might lead to alterations in cellular amino acid content is still unclear, although in – and efflux of various amino acids appear to be a transport-mediated phenomenon which could certainly be dependent upon such changes. In our investigation we observed increased levels of branched chain amino acids (BCAAs, i.e. valine, isoleucine and leucine) and basic amino acids (i.e. lysine and ornithine) were investigated following thiopentone treatment. Our results suggest that pH changes may have associated with these effects. Indeed, various investigations described that BCAAs as well as basic amino acids are involved in mechanisms of intracellular buffering (Lofberg et al., 1997; Bailey et al., 1996). In liver cells an intracellular alkanization following activation of Na^+/H^+-exchange led to an increase in intracellular leucine concentration (Mitsumoto and Mohri, 1986). Furthermore, especially basic amino acids are capable of carrying an extra positive charge over a wide range of physiological pH's and their presence may confer a mechanism of cellular buffering, too. Increased levels of basic and BCAAs may therefore represent a homeostatic reaction to changes in the acid/base balance induced by thiopentone.

High dose thiopentone treatment significantly decreases taurine levels in a dose-dependent manner. In PML-cells taurine accounts for approximately 60% of all intracellular amino acids (in our investigation the intracellular taurine concentrations amounted to ≈35–40 pMol per PML-cell) and these high intracellular concentrations are maintained against a high cell-plasma concentration gradient (Learn et al., 1990). The fact that taurine is present in abundance in PML-cells may draw attention to its role as an important intracellular osmoregulator and the results of our study may point to changes in PML osmoregulation following thiopentone treatment (Schousbõe and Pasantes-Morales, 1992). As found in investigations, thiopentone treatment seems to stimulate Na^+/H^+-exchange with the result of intracellular alkalization as well as increases in PML-cell volume. Cellular swelling as well as alterations in intracellular pH and Ca^{2+}-turnover following thiopentone seems to activate cellular volume regulation processes and particulary taurine transport. Fugelli et al. (1995) described that taurine is an important osmoeffector during regulatory volume decreases (RVD). Moreover, Olson et al. (1998) observed that net efflux of anionic taurine abandons a proton inside the cell thus lowering intracellular pH. Furthermore in our study we also observed increased levels of serine, glycine and alanine (so-called "neutral amino acids") which are known to be important organic osmolytes in cellular volume regulation processes, too (Chen and Kempson, 1995). Especially under conditions of both enhanced cellular accumulation of inorganic osmolytes and the concurrent changes in intracellular osmolality influx of neutral amino acids has been found. It might be important that both the decrease in taurine concentrations as well as the increase in intracellular neutral amino acids were accompanied by increases in aspartate concentrations. The interesting thing about these findings is that in heart cells hypoosmotically activated transport of amino acids is also associated with increased production of aspartate which may impart osmoprotection to the

stressed cells (Rasmusson et al., 1993). On the other hand glutamine is not released under conditions of cell swelling and shrinking. Altogether, the results of our investigation suggest an intracellular homeostasis where overall changes in intracellular acid/base buffering as well as cellular volume regulation processes are balanced by changes in intracellular taurine levels. This has significant consequences for PML cell function processes – especially regarding antibacterial host defense mechanisms – since taurine has been suggested to be directly involved in important leucocyte immune functions. Taurine influences leucocyte function in vivo and in vitro and seems to be involved in neutrophil-mediated host-defense. Additionally, studies suggest that functional activity of cells depends strongly by intracellular taurine and a protective effect of taurine on cellular function has been suggested, too (Stapleton et al., 1998). Masuda et al. (1986) found that the bactericidal capacity against Escherichia coli and phagocytotic activity in vivo was strengthened as the concentration of taurine in the serum increased with the term of its administration. Taurine deficiency has been found to exert deleterious effects on the functions of PML and B-lymphocytes in cats fed a prolonged taurine-deficient diet, too (Schuller-Levis et al., 1990). Concerning our findings there is an important reason to suppose that reduced immunocompetence of PML arises from the attemps to respond homeostatically to the anesthetic treatment.

As further important findings in our study we observed significant changes in free intracellular glutamine and glutamate concentrations following thiopentone treatment in PML. Glutamine has important and unique metabolic functions and in the last decade increasing evidence suggests that glutamine may be an important substrate especially for rapidly dividing and immunocompetent cells. It has been observed that high rates of glutamine utilisation by cells of the immune system serve to maintain a high intracellular concentration of both nitrogen and carbon for the synthesis of macromolecules and amino acids as well as glutaminolysis is an oxidative fuel for energy production (Newsholme and Calder, 1997; Castell et al., 1994). Consequently, the role of the high rate of glutaminolysis in PML and other rapidly dividing cells may be identical to that of glycolysis: providing ideal conditions for the precise and sensitive control of the rate of use of the intermediates of these pathways for biosynthesis when required. Mobilisation and activation of cells of the immune system, such as PML, may result in an increased rate of utilization of glutamine during cellular activity respectively utilization of glutamine may be decreased on account of PML inhibition following thiopentone (Roth et al., 1996). Indeed, our results suggest that one of the first steps of glutamine metabolism and/or significant decreases in glutamate transport which could be seen following intracellular alkanization (Dinkelborg et al., 1995), too to produce glutamate – which is the key amino acid in nitrogen turnover – may be decreased and might reflect therefore a lower efficiency of the glutaminolytic pathway in PML which may lead to a reduced function of these cells.

It seems unlikely that changes in important PML cell functions following thiopentone are due to changes in protein synthesis that are secondary to

alterations in intracellular amino acids. In our study we found dose-dependent alterations of concentrations of a combination of three different free intracellular amino acids such as: threonine, valine (essential amino acids) and histidine which in multiple regression analysis were selected to predict intracellular protein synthesis in adults (Metcoff, 1986). Indeed, former findings suggest that both alterations in cellular immune functions as well as changes in cellular hydration state are important factors controlling protein turnover. In rats, a close relationship between cell swelling and shrinking and the corresponding inhibition of protein degradation was found, regardless wether the cell volume was modified by amino acids or various osmolytes (Haussinger et al., 1993; Vom Dahl and Haussinger, 1996). Furthermore impaired PML functions following thiopentone influences generation of reactive oxidant products, too. Hazen et al. (1998) observed that neutrophils employ the enzyme myeloperoxidase – secreted in the course of activation – to oxidize amino acids to yield a family of reactive aldehydes which represent a product of reactive oxidant species. Stimulated human neutrophils likewise generated these aldehydes from all classes of amino acids. Logically, Stringer et al. (1995) described that inhibition of protein biosynthesis in human neutrophils resulted in a decreased ability to generate oxidants during respiratory burst. Additionally we found increased levels of free intracellular methionine following thiopentone treatment, which is known to be involved in the initiation states of protein biosynthesis, too. However concerning our results, further studies are necessary (such examinations currently are underway in our laboratory) to examine the particular effects of thiopentone on intracellular protein synthesis.

In conclusion: for the first time the effects of thiopentone on free intracellular amino acid metabolism in PML – related to single cell numbers – has been investigated using new, highly advanced, PML-separation and high-performance liquid chromatography techniques. Levels of important free intracellular amino acids were significantly changed in a dose-dependent manner and point to new biochemical mechanisms which are accompanied with thiopentone-induced changes in PML function. These mechanisms may involve changes in amino acid transport, intracellular acid/base balance, cellular volume regulation processes and protein turnover following short acting barbiturate treatment. Furthermore, alterations in intracellular taurine and glutamine/glutamate turnover might directly affect PML immune competence respectively anesthetic-induced changes in cellular host defense mechanisms.

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