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Benzodiazepine receptor-dependent modulation of neutrophil (PMN) free amino- and α-keto acid profiles or immune functions

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Summary. We have examined the effects of midazolam, Ro 5-4864 (agonist for "peripheral" [p] benzodiazepine receptors [BR]), PK 11195 (antagonist for pBR), flumazenil (antagonist for "central" BR), naloxone (antagonist for opiate receptors) and the combination of midazolam and Ro 5-4864, PK 11195, flumazenil or naloxone on intracellular amino- and α-keto acids and the immune function markers superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and released myeloperoxidase (MPO) activity in neutrophils (PMN). Only midazolam and Ro 5-4864 led to significant changes in the dynamic PMN free amino- and α-keto acid pools. Concerning PMN immune function markers, midazolam and Ro 5-4864 significantly decreased O_2^- and H_2O_2 formation and released MPO. When midazolam and Ro 5-4864 were applied together they appeared to act additively. Pre-incubation with PK 11195 partially neutralized the midazolam effects whereas flumazenil or naloxone showed no effects. We therefore believe that pBR are involved in the signal transmission of anesthetic-induced cellular metabolic changes in PMN.

Keywords: Benzodiazepine receptors – Neutrophil – Amino acids – α-Keto acids – Immune function

Introduction

Neutrophilic polymorphonuclear granulocytes (PMN) represent the most mobile and numerically superior cellular components of the natural non-specific immune defense of the human body (Gabrilovich, 1999). They accumulate at sites of traumatic tissue damage and infections in response to proinflammatory and chemotactic factors as well as various other mediators of the immune system (e.g. antibodies, complement factors and cytokines) (Witko-Sarsat et al., 2000). They also tackle pathogenic microorganisms, other cells recognized as pathogenic as well as other agents using a fascinating arsenal of defense mechanisms (Winterbourn et al., 2000). Neutrophilic granulocytes are as such equipped with specific properties that enable the active reaching, recognition, and subsequent annihilation of the pathogens, their so-called "raison d'être" (Burg and Pillinger, 2001). The ability to activate the PMN non-specifically, as well as the diverse combination of highly efficient defensive functions (phagocytosis, degranulation and oxidative activity), determine the essential role of these cells in conveying the body's immune response (Cohen, 1994). As a possible consequence of PMN malfunction, bacterial infection may develop and contribute to patient morbidity and mortality (Bihari, 2002; Takrouri, 2002; Waitzberg et al., 2003). Ideally, the provision of optimal preconditions for PMN cells exists as an undisturbed function of major immunocompetent PMN biochemical pathways. Increasing evidence suggests that free intracellular amino acid and α-keto acid turnover is particularly important for the metabolic and physiological state of PMN as well as the special functions in the inflammatory response of these cells (Moinard et al., 2002a, b; Metcoff, 1986; Metcoff et al., 1987; Mühling et al., 2003, 1999).

Recent findings have pointed towards medication as a major influential factor on important immunological functions in PMN (Heller et al., 1998; Krumholz et al., 1993). Unfortunately, this also applies to remedies that are often applied for sedation and analgesia of intensive-care

patients on a relatively long-term and continuous basis (Krumholz et al., 1995a, b). Although all preparations applied to critically ill patients (including sedatives and hypnotics from anesthesia and intensive-care medicine) require a very precise monitoring regarding their immunomodulatory and metabolic-physiological influence, only a few investigations on the influence of such agents on PNM intracellular amino- and α-keto acid turnover and important PMN immune functions have been carried out (Mühling et al., 2000a, b, 2001a, b, 2002). Some investigators have reported that the benzodiazepine midazolam, which also plays an important part in modern anesthesia as well as in intensive care medicine, impairs PMN function even at clinically relevant concentrations (Davidson et al., 1995; Nishina et al., 1998; Tian et al., 1994; Weiss et al., 1993). These effects do not necessarily lead to perioperative immunosuppression, but they may have the potential to compromise already depressed host defense mechanisms even further. Moreover, several experimental and clinical studies support a key role for benzodiazepine receptors of the peripheral type in mediating the metabolic and immunological effects of benzodiazepines (Cosentino et al., 2000; Marino et al., 1998, 2001; Finnerty et al., 1991; Laghi et al., 1987). Peripheral benzodiazepine receptors differ from the central benzodiazepine receptors linked to the GABA-receptor-chloride channel complex in that they show a different distribution, molecular structure and pharmacological sensitivity. The peripheral benzodiazepine receptors bind selectively to the agonist Ro-5-4864 and the antagonist PK 11195, and do not bind to flumazenil, which displays a high affinity for central-type benzodiazepine receptors (Cosentino et al., 2000; Marino et al., 1998, 2001; Finnerty et al., 1991; Laghi et al., 1987). Peripheral benzodiazepine receptors are expressed with high density on human PMN and circumstantial evidence suggests that they may play a role in benzodiazepine-induced modulation of neutrophil function.

The goals of this study are therefore:

1) To document the effects of *midazolam* (as a possible example of maleficient pharmacological stress) on PMN free intracellular amino- and α-keto acid concentrations as well as on the activities of released myeloperoxidase (MPO), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) formation (as markers of PMN function and in order to show possible parallels with changes in PMN amino acid concentrations).
2) To investigate the effects of *Ro-5-4864* (agonist to ''peripheral'' benzodiazepine receptors), *PK 11195* (antagonist to ''peripheral'' benzodiazepine receptors), *flumazenil* (antagonist to ''central'' benzodiazepine receptors) and *naloxone* (antagonist to opiate receptors) on PMN free intracellular amino- and α-keto acid concentrations and on PMN immune functions (MPO, O_2^-, $H_2O_2^-$).
3) To explore the effects of *Ro-5-4864*, *PK 11195*, *flumazenil* and *naloxone* (pre-incubated for 10 min) and *midazolam* on PMN amino- and α-keto acid concentrations and important PMN immune functions in order to establish whether an agonist and antagonist to ''peripheral'' benzodiazepine receptors as well as antagonists to ''central'' benzodiazepine and opiate receptors may influence any of the *midazolam*-induced effects.
4) To examine whether there is a critical duration of exposure required to produce any significant effects.

Materials and methods

The study was approved by the local ethics committee of the Justus Liebig University, Giessen. Ten men between 24 and 37 years (31 ± 3.9) with an average height of 179.0 cm (range 171–187) and weight of 79.1 kg (range 73–91) were selected: those men with metabolic (diabetes etc.), cardiopulmonary, neurological or allergic diseases or men taking drugs were excluded. Whole blood samples (lithium-heparinate plastic tubes) were withdrawn between 08:00 and 09:00 (after 10 hours of fasting) considering circadian variations.

Midazolam

To document any dose- and duration of exposure-dependent effects, PMN were incubated with different midazolam concentrations (*0*, *40* and *100 ng/ml*, *1 μg/ml*) for 10, 30, 60 and 120 min. The selected midazolam concentrations corresponded to a) minimal effective plasma concentrations at which an incipient clinical effectiveness has been described, b) effective or also optimal plasma concentrations, and c) 10-fold the clinically achieved plasma concentrations (Davidson et al., 1995; Heine et al., 1996; Heller et al., 1998; Weiss et al., 1993).

Receptor agonists and -antagonists

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

1. *Ro-5-4864* (agonist to ''peripheral'' benzodiazepine receptors): *1 and 100 μM* [7-Chloro-5-(4′-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine-2; Sigma, Deisenhofen]
2. *PK 11195* (antagonist to ''peripheral'' benzodiazepine receptors): *1 and 100 μM* [1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolincarboxamide; Sigma, Deisenhofen]
3. *Flumazenil* (antagonist to ''central'' benzodiazepine receptors): *1 and 100 μM* [Anexate®, Hoffmann La Roche, Grenzach-Wyhlen]
4. *Naloxone* (antagonist to opiate receptors): *1 and 100 μM* Narcanti®, Du Pont Pharma, Bad Homburg

PMN were incubated with agents for 10 and 120 min to examine the dynamics of response to these agents. The final concentrations of Ro-5-4864, PK 11195, flumazenil or naloxone selected corresponded – in accordance to previous work – with our own preliminary examinations (not published).

Midazolam + receptor agonists and -antagonists

The following concentrations were tested in addition to the control:

Midazolam (100 ng/ml and 1 μg/ml):
+ *Ro-5-4864* (100 μM)
+ *PK 11195* (100 μM)
+ *Flumazenil* (100 μM)
+ *Naloxone* (100 μM).

Midzolam was added after a 10 min pre-incubation of Ro-5-4864, PK 11195, flumazenil or naloxone. This mixture was incubated again for another 120 minutes.

Solutions 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 ornithine concentrations were as described above) at 37°C using a shaking water bath. Corresponding volumes of HBSS were added to the control tubes. Before further processing, all fractions were immediately cooled in an ice water bath at 4°C and 100 μg/ml phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin, as well as 10 μg/ml antipain (all acquired from Sigma, USA) were added to each plastic heparin tube before the blood samples; these additions served to inhibit proteases.

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

Precise details of our PMN-separation technique have been described previously (Mühling et al., 2003, 1999). This method is a further development of the methods described by Eggleton et al. (1989) and Krumholz et al. (1995, 1993) which allows a very rapid and selective enrichment of PMN while preserving high cellular viability and integrity from very small quantities of whole blood.

Chromatographic amino and α-keto acid analysis

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

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

Superoxide anion production was measured by reduction of cytochrome C. Cytochrome C (100 mg, type IV, Sigma, Deisenhofen, Germany) was dissolved in 30 ml PBS® glucose buffer (Gibco, Karlsruhe, Germany). The solution was portioned and stored at −20°C. Opsonized zymosan (Sigma, Deisenhofen, Germany) was used to stimulate PMN. It was evoked by incubating 100 mg zymosan with 6 ml pool serum for 30 min at 37°C. After washing with saline and centrifuging at 350 × g (10 min), opsonized zymosan was resuspended in 10 ml PBS® glucose buffer, portioned and stored at −20°C. Whole blood was incubated with test solutions described above. These preparations were then incubated for 10, 30, 60 and 120 min at 37°C (shaking water bath). The PMN were thereafter isolated using a modified PMN separation technique as mentioned above. After stepwise (15 min and 5 min) centrifugation procedures (350 × g, 20°C) as well as careful lysis of a few erythrocytes contaminating the pellet, the PMN cells were resuspended by adding diluted PBS® (Gibco, Karlsruhe, Germany) stock buffer. After administration of 7 ml PBS® stock buffer, the tubes were centrifuged at 350 × g for 5 min (20°C). The supernatant was decanted. Samples with a PMN purity <96% and those with more than 4% dead cells were discarded. The PMN concentration (0.8×10^6/mL) was adjusted by adding PBS® containing 9.99 g/l glucose (Merck, Darmstadt, Germany). After PMN isolation, 500 μl zymosan, 150 μl pool serum, 250 μl cytochrome C and 500 μl isolated PMN suspension (0.8×10^6/ml) and again test solutions were poured into a test tube. A preparation containing 500 μl buffer instead of zymosan was used for zero adjustment. After incubation for 15 min at 37°C, the reaction was stopped by putting the test tube into ice water. After centrifugation (350 × g; 3 min, 4°C) extinction of the supernatant was measured photometrically (546 nm; Digitalphotometer 6114S®; Eppendorf, Germany). The amount of superoxide anions measured resulted from the extinction coefficient of cytochrome C (Rick 1977). All control samples were prepared, incubated and measured in the same way.

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

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

Statistical analysis

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

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

Results

The free intracellular 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 (Krumholz et al., 1995, 1993; Mühling et al., 2003, 1999).

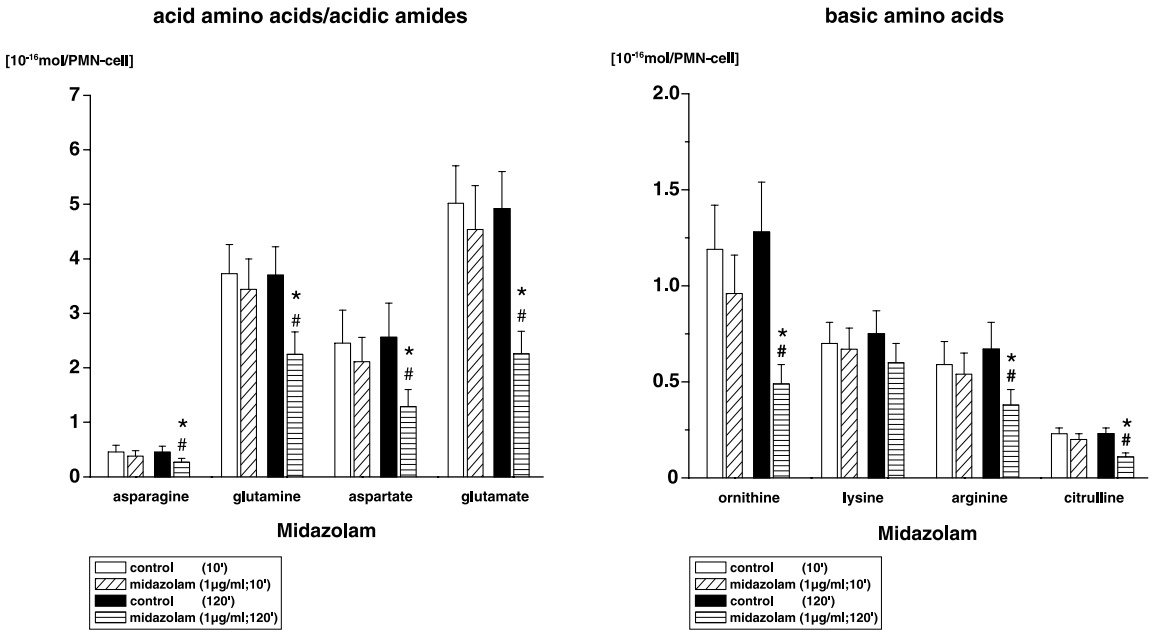

Fig. 1. Effects of *midazolam* (1 μg/ml) incubated with whole blood for 10 and 120 min on free intracellular *acidic amino acid*, *acidic amide* and *basic amino acid* concentrations in PMN (10^{-16} Mol per PMN-cell; mean $\pm$ SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

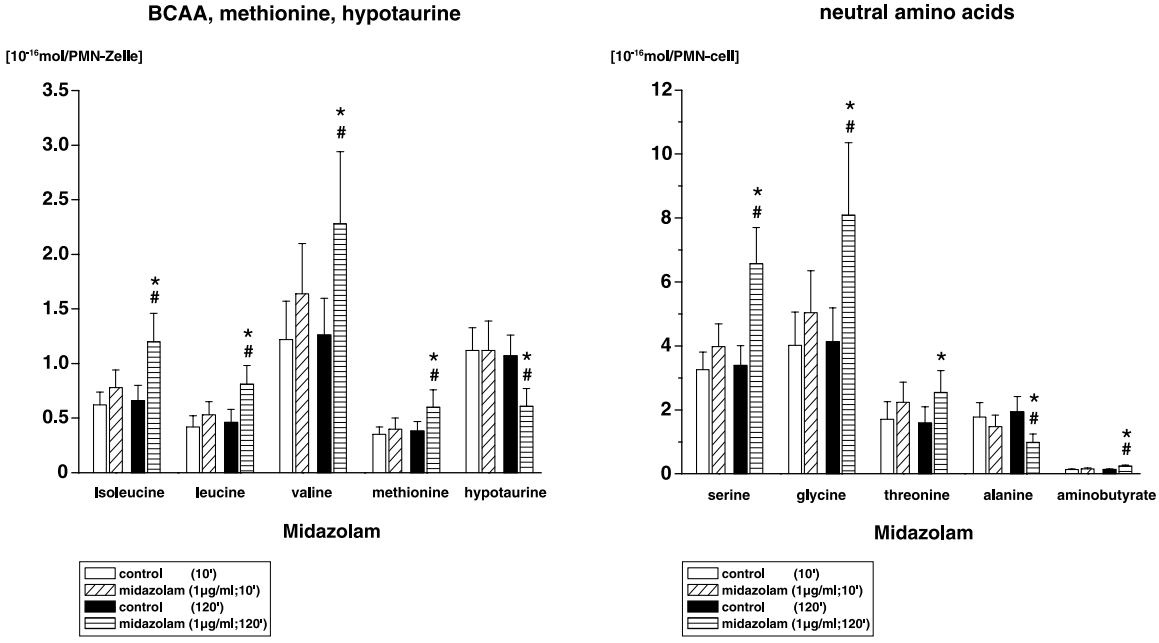

Fig. 2. Effects of *midazolam* (1 μg/ml) incubated with whole blood for 10 and 120 min on free intracellular *branched chain amino acid* (*BCAA*), *methionine*, *hypotaurine* and *neutral amino acid* concentrations in PMN (10^{-16} Mol per PMN-cell; mean $\pm$ SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

Midazolam

Effects of midazolam on the free amino acid pool in PMN

Concentrations of free intracellular amino acids were unaffected by 40 ng/ml midazolam (PMN incubation for 10, 30, 60 or 120 min). Following ≥100 ng/ml midazolam (PMN incubation for ≥30 min), significant decreases in PMN asparagine, glutamine, aspartate, glutamate, ornithine, arginine, citrulline (Fig. 1) as well as in PMN alanine, hypotaurine (Fig. 2) and taurine (Fig. 3) were observed. Moreover, midazolam (≥100 ng/ml; PMN incubation for ≥30 min) significantly increased PMN isoleucine, leucine, valine, methionine, serine, glycine, threonine, α-aminobutyrate (Fig. 2) and histidine (data not shown). PMN free lysine, tyrosine, tryptophane and phenylalanine profiles remained unaffected.

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

Following a short duration of midazolam exposure (10 min; 40 and 100 ng/ml, 1 μg/ml) concentrations of free intracellular α-keto acids remained unaffected. In the presence of ≥100 ng/ml midazolam (PMN incubation for

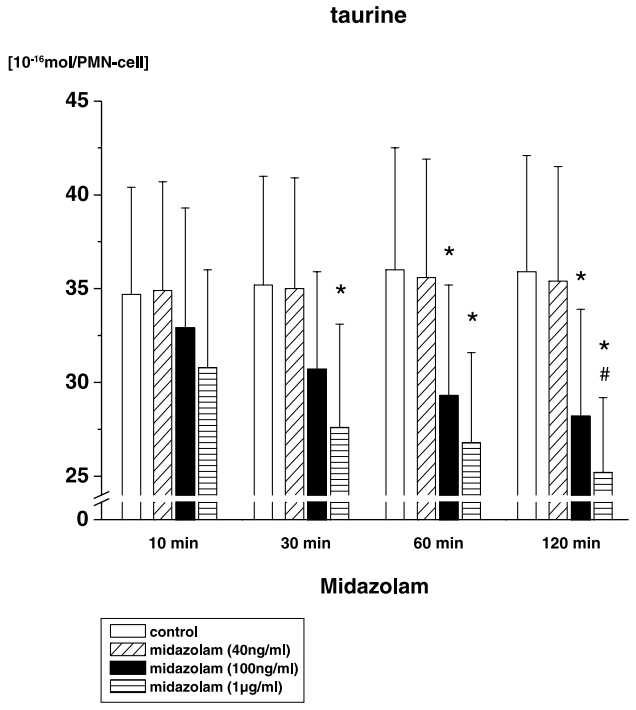

Fig. 3. Effects of *midazolam* (40 ng/ml, 100 ng/ml and 1 μg/ml) incubated with whole blood for 10, 30, 60 and 120 min on free intracellular *taurine* concentrations in PMN (10^{-16} Mol per PMN-cell; mean ± SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

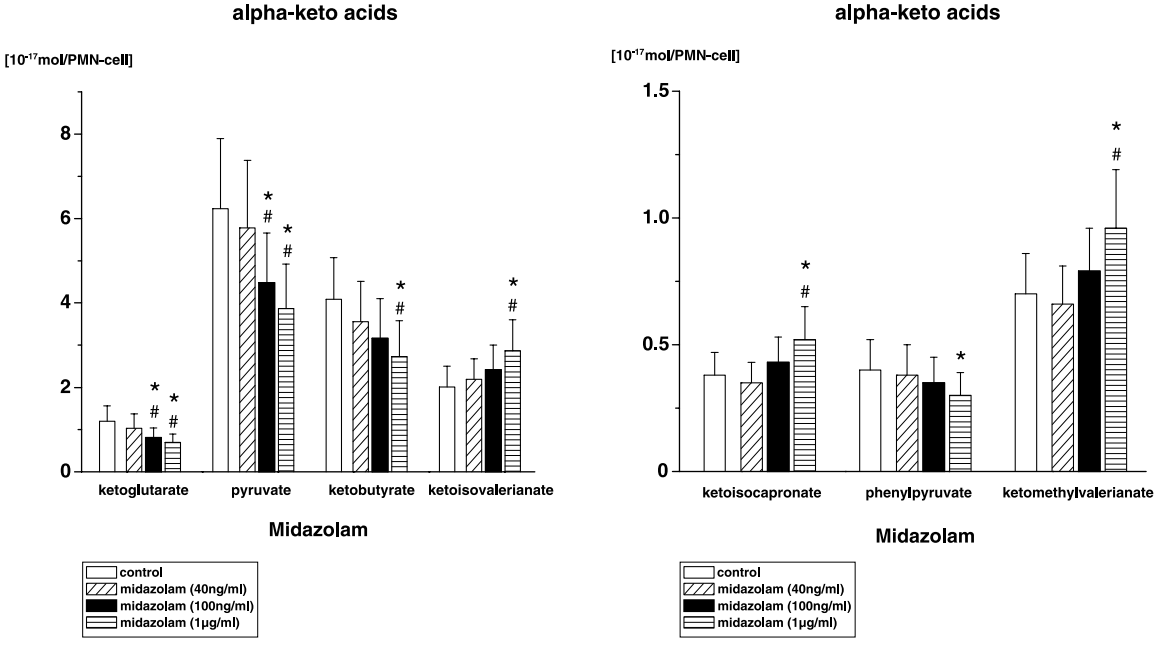

Fig. 4. Effects of *midazolam* (1 μg/ml) incubated with whole blood for 120 min on free intracellular *α-keto acid* (*α-ketoglutarate*, *pyruvate*, *α-ketobutyrate*, *α-ketoisovalerianate*, *α-ketoisocapronate*, *p-hydroxy-phenylpyruvate* and *α-keto-β-methylvalerianate*) concentrations in PMN (10^{-17} Mol per PMN-cell; mean ± SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

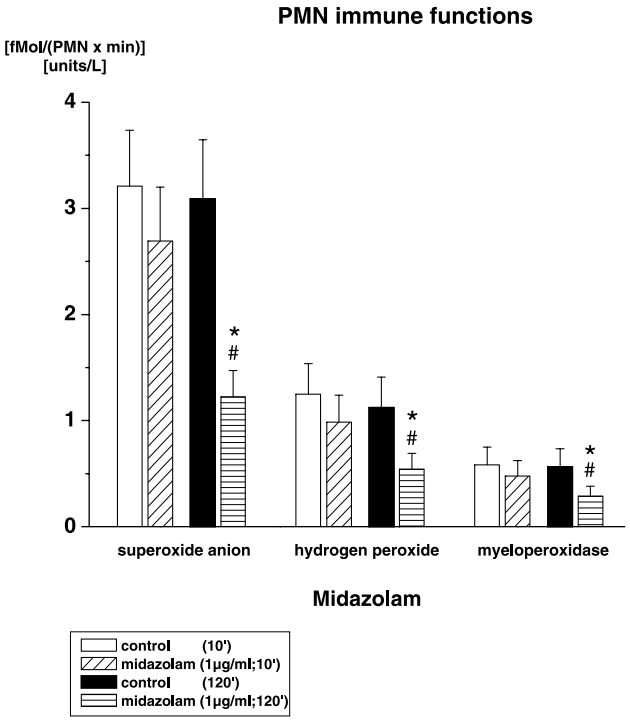

Fig. 5. Effects of *midazolam* (40 ng/ml, 100 ng/ml and 1 μg/ml) incubated with whole blood for 10 and 120 min on PMN superoxide anion [fMol O_2^-/(PMN $\times$ min)] and hydrogen peroxide production [fMol H_2O_2/(PMN $\times$ min)] and on PMN myeloperoxidase activity ([Units/l supernatant]; mean $\pm$ SD; n = 10). $^*p \leq 0.05$ versus control values; $^\#p \leq 0.05$ 120 min versus 10 min

120 min), significant decreases in PMN α-ketoglutarate, pyruvate, α-ketobutyrate, p-hydroxy-phenylpyruvate and increases in α-ketoisovaleriate, α-ketoisocapronate and α-keto-β-methylvaleriate were found (Fig. 4).

Effects of midazolam on oxidative response and myeloperoxidase activity

All PMN immune functions tested were unaffected up to 40 ng/ml midazolam. In the presence of higher midazolam doses ($\geq$100 ng/ml; $\geq$60 min), PMN superoxide anion, hydrogen peroxide production and MPO activity decreased significantly (Fig. 5).

Receptor agonists and -antagonists

Effects of Ro 5-4864, PK 11195, flumazenil or naloxone on the free amino acid pool in PMN

Concentrations of free intracellular amino acids were unaffected by PK 11195, flumazenil or naloxone. Relevant changes in free intracellular amino acids only occurred after whole blood incubation with Ro 5-4864 ($\geq$1 μM) for 120 min. Ro 5-4864 decreased PMN asparagine, glutamine, aspartate, glutamate, ornithine, arginine, citrulline (Fig. 6), alanine, hypotaurine (Fig. 7) and taurine (data not shown) and increased PMN isoleucine, leucine, valine, methionine, serine, glycine, threonine, α-aminobutyrate (Fig. 7) and histidine (data not shown). None of the other amino acids have been affected significantly.

Effects of Ro 5-4864, PK 11195, flumazenil or naloxone on the free α-keto acid pool in PMN

Only in the presence of Ro 5-4864 ($\geq$1 μM; PMN incubation for 120 min) were there significant decreases in

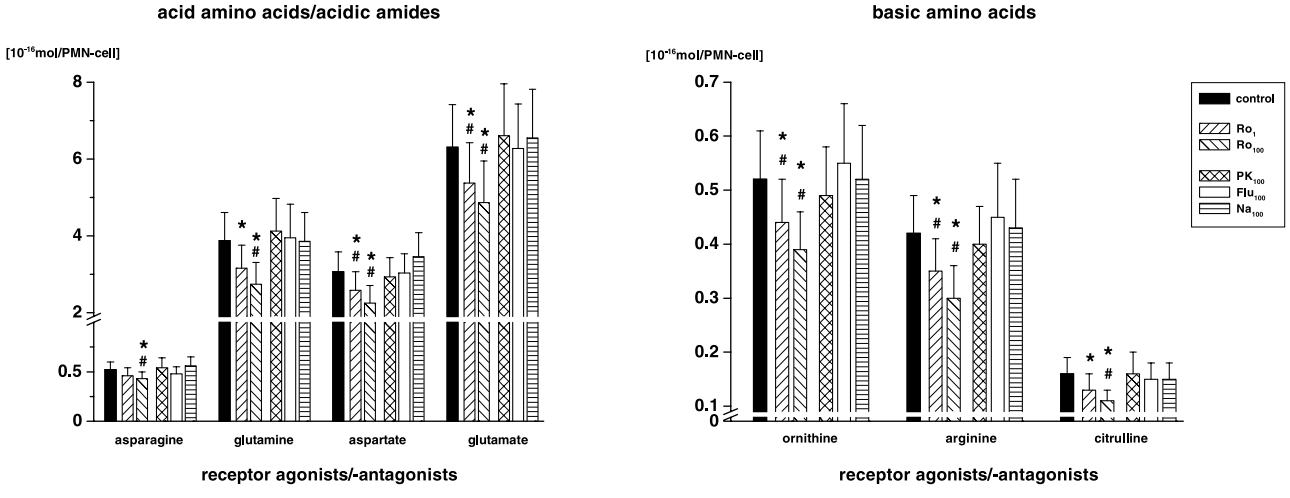

Fig. 6. Effects of *Ro 5-4864* [1 (Ro_1) und 100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] and *naloxone* [100 μM (Na_{100})] incubated with whole blood for 120 min on free intracellular *acidic amino acid*, *acidic amide* and *basic amino acid* concentrations in PMN (10^{-16} Mol per PMN-cell; mean $\pm$ SD; n = 10). $^*p \leq 0.05$ versus control values; $^\#p \leq 0.05$ 120 min versus 10 min

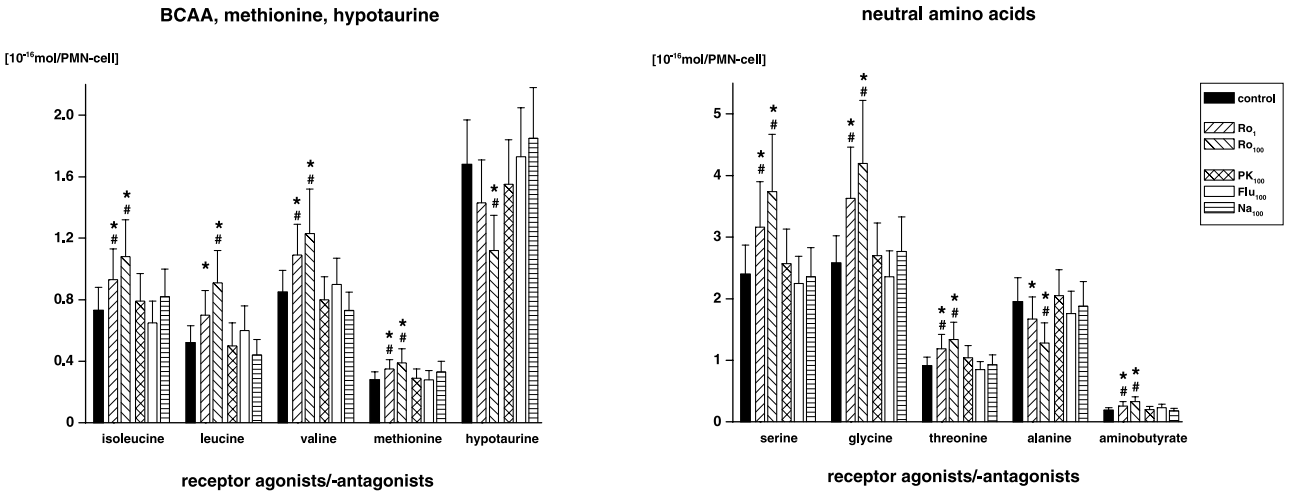

Fig. 7. Effects of *Ro 5-4864* [1 (Ro_1) und 100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] and *naloxone* [100 μM (Na_{100})] incubated with whole blood for 120 min on free intracellular *branched chain amino acid* (*BCAA*), *methionine*, *hypotaurine* and *neutral amino acid* concentrations in PMN (10^{-16} Mol per PMN-cell; mean $\pm$ SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

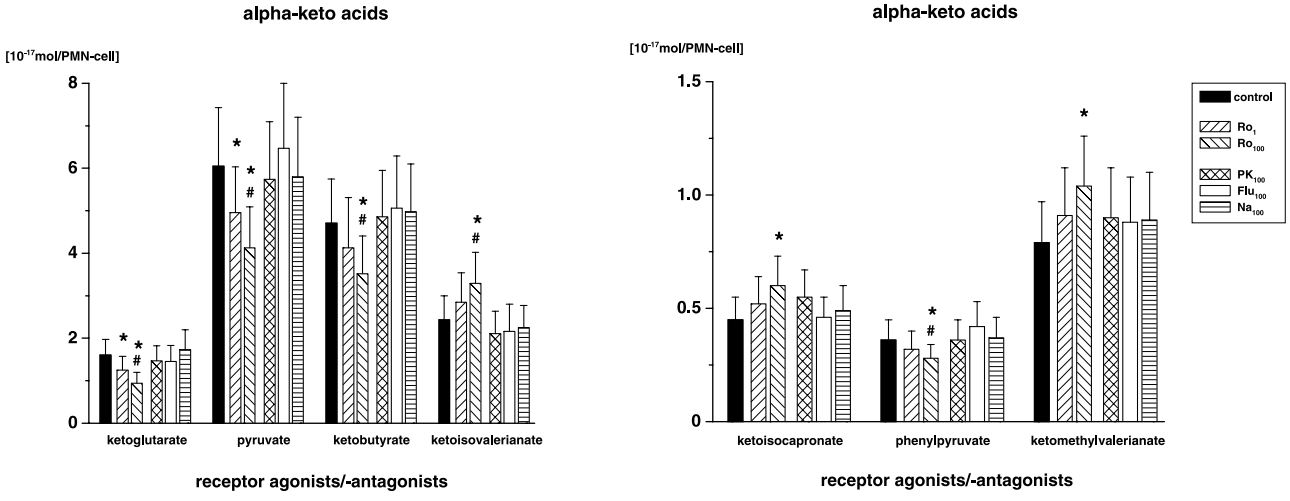

Fig. 8. Effects of *Ro 5-4864* [1 (Ro_1) und 100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] and *naloxone* [100 μM (Na_{100})] incubated with whole blood for 120 min on free intracellular α-*keto acid* (*α-ketoglutarate*, *pyruvate*, *α-ketobutyrate*, *α-ketoisovalerianate*, *α-ketoisocapronate*, *p-hydroxy-phenylpyruvate* and *α-keto-β-methylvalerianate*) concentrations in PMN (10^{-17} Mol per PMN-cell; mean $\pm$ SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

PMN α-ketoglutarate, pyruvate, α-ketobutyrate, p-hydroxy-phenylpyruvate and increases in α-ketoisovaleriate, α-ketoisocapronate and α-keto-β-methylvaleriate (Fig. 8).

Effects of Ro 5-4864, PK 11195, flumazenil or naloxone on oxidative response and myeloperoxidase activity

All PMN immune functions tested were unaffected by PK 11195, flumazenil or naloxone. In the presence of Ro 5-4864 ($\geq$1 μM; PMN incubation for 120 min), PMN superoxide anion, hydrogen peroxide production and MPO activity decreased significantly (Fig. 9).

Midazolam + receptor agonists and -antagonists

Effects of midazolam + Ro 5-4864, PK 11195, flumazenil or naloxone on free amino acid pool in PMN

Pre-incubation of Ro 5-4864 (100 μM; 10 min) followed by lower midazolam doses (100 ng/ml; PMN incubation

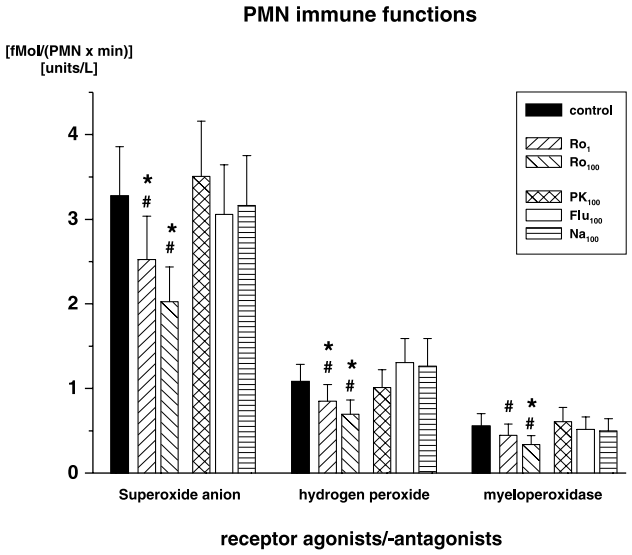

Fig. 9. Effects of *Ro 5-4864* [1 (Ro_1) und 100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] and *naloxone* [100 μM (Na_{100})] incubated with whole blood for 120 min on PMN superoxide anion [fMol O_2^-/(PMN $\times$ min)] and hydrogen peroxide production [fMol H_2O_2/(PMN $\times$ min)] and on PMN myeloperoxidase activity ([Units/l supernatant]; mean $\pm$ SD; n = 10). $^{\star}p \leq 0.05$ versus control values; $^{\#}p \leq 0.05$ 120 min versus 10 min

for 120 min) led to additional effects with further decreases in PMN aspartate, glutamate, ornithine, arginine, citrulline (Fig. 10), hypotaurine, taurine (data not shown), alanine (Fig. 11) and increases in isoleucine, leucine, valine, serine, glycine and threonine (Fig. 11) compared to the addition of midazolam alone. Pre-incubation with Ro 5-4864 followed by higher midazolam doses (1 μg/ml; PMN incubation for 120 min) did not show different results compared to the addition of midazolam alone (data not shown). PK 11195 (100 μM; pre-incubated for 10 min), followed by lower midazolam doses (100 ng/ml) completely neutralized free intracellular changes in PMN aspartate, glutamate, ornithine, arginine, citrulline (Fig. 10), hypotaurine, taurine (data not shown), alanine (Fig. 11), isoleucine, leucine, valine, serine, glycine, threonine (Fig. 11) and histidine (data not shown) compared to midazolam alone. At higher midazolam doses (1 μg/ml) PK 11195 led only to a partial restoration of the amino acid changes (data not shown) induced by midazolam. With flumazenil (100 μM; pre-incubated for 10 min) or naloxone (100 μM; pre-incubated for 10 min) + midazolam (0.1 and 1 μg/ml; PMN incubation for 120 min) treatment, free intracellular amino acids remained unaffected compared to midazolam alone (Figs. 10 and 11).

Effects of midazolam + Ro 5-4864, PK 11195, flumazenil or naloxone on free α-keto acid pool in PMN

In accordance with the amino acid results, pre-incubation of Ro 5-4864 (100 μM; 10 min) followed by lower midazolam doses (100 ng/ml; PMN incubation for 120 min) led to additional effects with further decreases in PMN α-ketoglutarate, pyruvate, α-ketobutyrate, p-hydroxy-phenylpyruvate and increases in α-ketoisovaleriate whereas α-ketoisocapronate, p-hydroxy-phenylpyruvate and

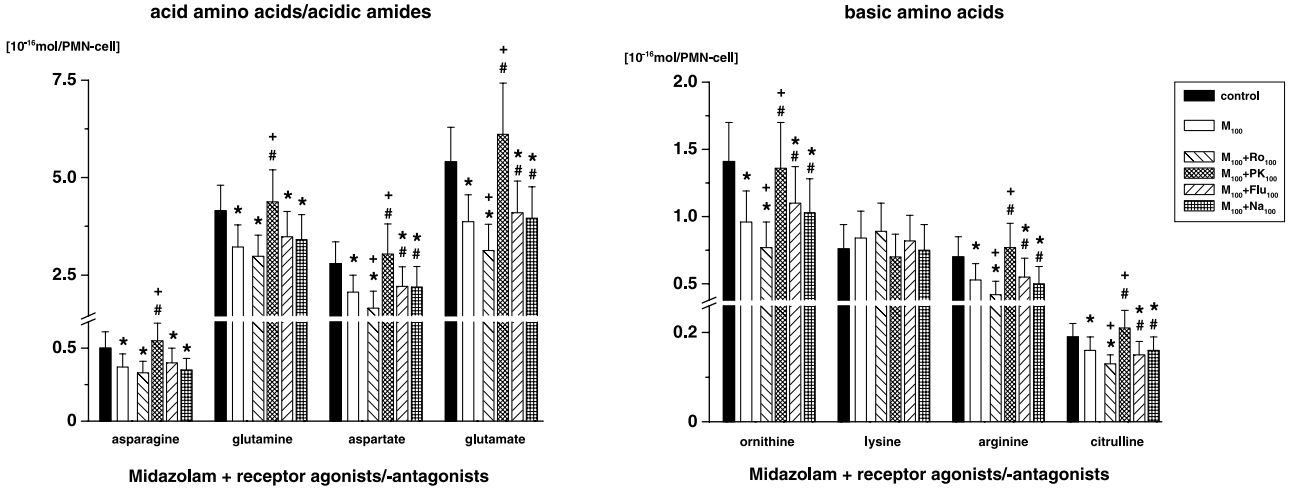

Fig. 10. Effects of *midazolam* [100 ng/ml (M_{100})] incubated with whole blood for 120 min combined either with *Ro 5-4864* [100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] or *naloxone* [100 μM (Na_{100})], pre-incubated for 10 min, on free intracellular *acidic amino acid*, *acidic amide* and *basic amino acid* concentrations in PMN (10^{-16} Mol per PMN-cell; mean $\pm$ SD; n = 10). $^{\star}p \leq 0.05$ versus control values; $^{+}p \leq 0.05$ versus M_{100}; $^{\#}p \leq 0.05$ versus $M_{100} + Ro_{100}$

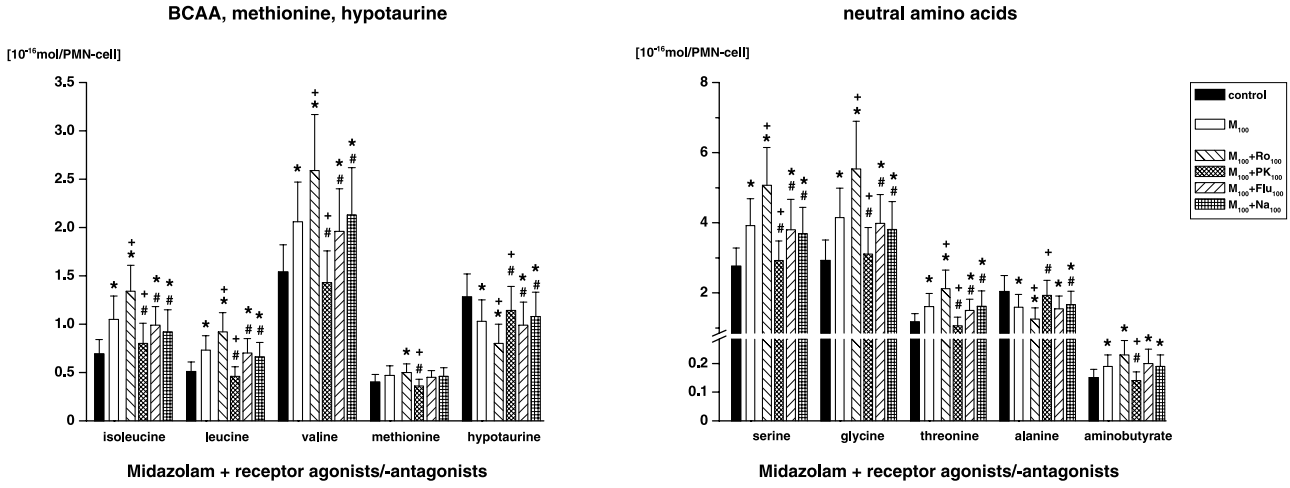

Fig. 11. Effects of *midazolam* [100 ng/ml (M_{100})] incubated with whole blood for 120 min combined either with *Ro 5-4864* [100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] or *naloxone* [100 μM (Na_{100})], pre-incubated for 10 min, on free intracellular *branched chain amino acid* (*BCAA*), *methionine*, *hypotaurine* and *neutral amino acid* concentrations in PMN (10^{-16} Mol per PMN-cell; mean ± SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{+}p \leq 0.05$ versus M_{100}; $^{\#}p \leq 0.05$ versus $M_{100} + Ro_{100}$

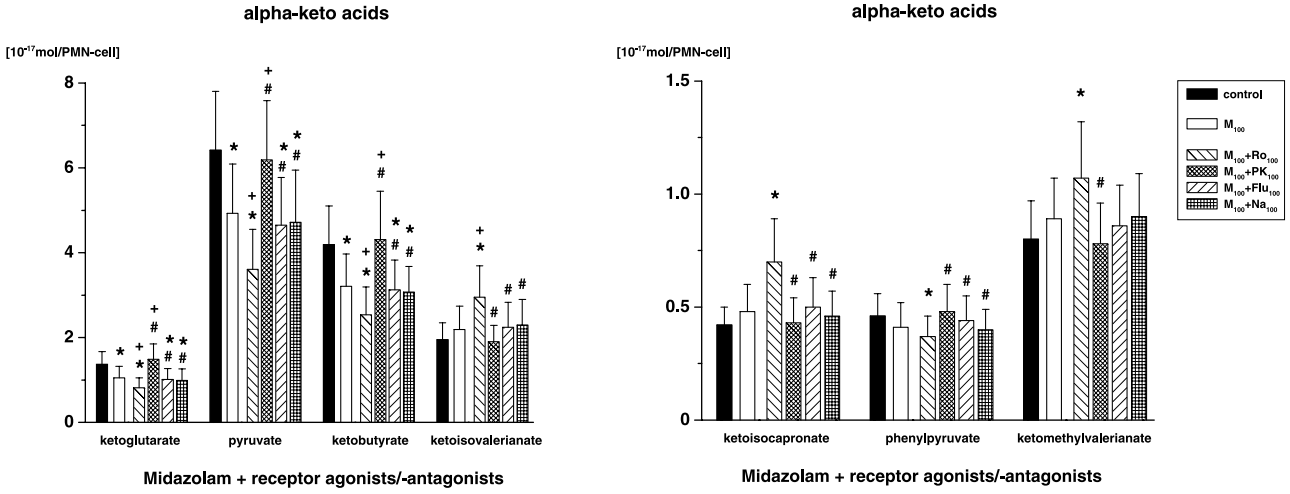

Fig. 12. Effects of *midazolam* [100 ng/ml (M_{100})] incubated with whole blood for 120 min combined either with *Ro 5-4864* [100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] or *naloxone* [100 μM (Na_{100})], pre-incubated for 10 min, on free intracellular *α-keto acid* (*α-ketoglutarate*, *pyruvate*, *α-ketobutyrate*, *α-ketoisovalerianate*, *α-ketoisocapronate*, *p-hydroxy-phenylpyruvate* and *α-keto-β-methylvalerianate*) concentrations in PMN (10^{-17} Mol per PMN-cell; mean ± SD; n = 10). $^{*}p \leq 0.05$ versus control values; $^{+}p \leq 0.05$ versus M_{100}; $^{\#}p \leq 0.05$ versus $M_{100} + Ro_{100}$

α-keto-β-methylvaleriate (Fig. 12) remained unaffected compared to midazolam alone. Pre-incubation with Ro 5-4864 followed by higher midazolam doses (1 μg/ml; PMN incubation for 120 min) did not show different results compared to the exclusive addition of midazolam (data not shown). PK 11195 (100 μM; pre-incubated for 10 min), followed by lower midazolam doses (100 ng/ml), completely neutralized free intracellular α-ketoglutarate, pyruvate and α-ketobutyrate changes induced by midazolam alone (Fig. 12). At higher midazolam doses (1 μg/ml), pre-incubation of PK 11195 led only to a partial inversion of α-keto acid changes (all α-keto acids measured; data not shown) induced by midazolam. Following flumazenil (100 μM; pre-incubated for 10 min) or naloxone (100 μM; pre-incubated for 10 min) + midazolam (0.1 and 1 μg/ml; PMN incubation for 120 min) treatment, free intracellular amino acids remained unaffected compared to midazolam alone (Fig. 12).

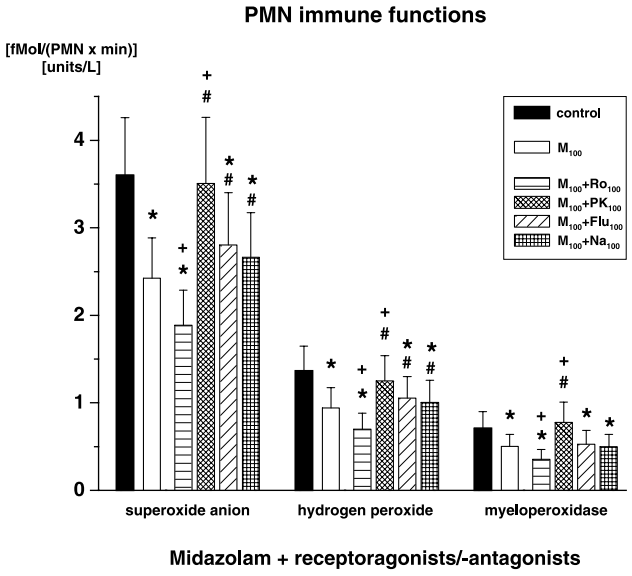

Fig. 13. Effects of *midazolam* [100 ng/ml (M_{100})] incubated with whole blood for 120 min combined either with *Ro 5-4864* [100 μM (Ro_{100})], *PK 11195* [100 μM (PK_{100})], *flumazenil* [100 μM (Flu_{100})] or *naloxone* [100 μM (Na_{100})], pre-incubated for 10 min, on PMN superoxide anion [fMol O_2^-/(PMN $\times$ min)] and hydrogen peroxide production [fMol H_2O_2/(PMN $\times$ min)] and on PMN myeloperoxidase activity ([Units/l supernatant]; mean $\pm$ SD; n = 10). $^*p \leq 0.05$ versus control values; $^+p \leq 0.05$ versus M_{100}; $^\#p \leq 0.05$ versus $M_{100} + Ro_{100}$

Effects of midazolam + Ro 5-4864, PK 11195, flumazenil or naloxone on oxidative response and myeloperoxidase activity

Pre-incubation of Ro 5-4864 (100 μM; 10 min) followed by midazolam (0.1 and 1 μg/ml; PMN incubation for 120 min) led to additional effects with further decreases in PMN superoxide anion, hydrogen peroxide production and MPO activity (Fig. 13). PK 11195 (100 μM; pre-incubated for 10 min) completely (0.1 μg/ml; PMN incubation for 120 min) or partially (1 μg/ml; PMN incubation for 120 min) neutralized changes in immune function markers induced by midazolam alone (Fig. 13). Following flumazenil (100 μM; pre-incubated for 10 min) or naloxone (100 μM; pre-incubated for 10 min) + midazolam (0.1 and 1 μg/ml; PMN incubation for 120 min) treatment no relevant changes in PMN function compared to midazolam alone occurred (Fig. 13).

Discussion

Clinically relevant and higher midazolam concentrations significantly decreased intracellular glutamine, glutamate, aspartate, asparagine, arginine, ornithine, citrulline, alanine, hypotaurine and taurine levels (requiring $\geq$30 min exposure at the lower concentration), and increased the levels of other important amino acids (i.e. histidine, isoleucine, leucine, valine, methionine, serine, glycine, threonine). Only the aromatic acids and lysine were not influenced. Equally, clear reductions in PMN α-ketoglutarate, pyruvate, α-ketobutyrate, p-hydroxy-phenylpyruvate and increases in α-ketoisovaleriate, α-ketoisocapronate and α-keto-β-methylvaleriate content were also shown. Parallel to these changes, dose and exposure-time dependent decreases in superoxide anion and hydrogen peroxide generation as well as PMN myeloperoxidase activity were also found. Important findings of our study show that mediazolam incubation of whole blood significantly decreased PMN intracellular taurine and hypotaurine profiles; both these amino acids account for approximately 55–60% of all PMN free intracellular amino acids ($\approx 30–40 \cdot 10^{-16}$ Mol per PMN-cell), which may draw attention to essential changes in PMN amino acid content as well as PMN osmoregulation (Fugelli and Thoroed, 1990; Law, 1991). High intracellular taurine and hypotaurine concentrations are maintained against a high cell-plasma concentration gradient ($\approx$100–200:1) (Learn et al., 1990). Interestingly, decreases in intracellular taurine concentration are closely followed by increases in PMN neutral amino acid levels which are also known to balance significant changes in intracellular amino acid content as well as impaired osmoprotection in various cells (Chen et al., 1994; Chen and Kempson, 1995). Our results therefore suggest that 1) neutral amino acid levels may be related to intracellular taurine levels that in so doing balance intracellular amino acid content and 2) the changes in taurine may indeed reflect an osmoregulatory reaction to the alteration in amino acid content (i.e. the increase in amino acids followed an induced decrease in taurine levels). This may also have interesting immunonutritive consequences. Redmond et al. (1998), but also Stapleton and co-workers (1998), described that intracellular taurine in particular may be effective at ameliorating defective phagocytic as well as proinflammatory cell microbicidal capacity and in maintaining cell viability associated with aging of PMN and may therefore strengthen the immunological competence of PMN. Moreover, the specific effects of midazolam on glutamine, glutamate, and α-ketoglutarate probably reflect an important change in the metabolic state of PMN cells, since it has been established that glutamine and glutamate, which enters the tricarboxylic acid cycle (TCA) via α-ketoglutarate, are important nutrients for leukocytes, even while resting (Curi et al., 1999; Furukawa et al., 1997; Moinard et al., 1999). The role of glutaminolysis in rapidly

dividing cells is considered to be in the provision of both nitrogen and carbon for the synthesis of macromolecules (i.e. RNA, DNA), intermediates of major cellular biosynthetic pathways and energy (Saito et al., 1999; Newsholme, 1996; Newsholme and Calder, 1997; Newsholme, 2001). Therefore glutaminolysis is directly related to the specific functions of leukocytes such as PMN in the inflammatory response and we therefore suggest that changes in PMN glutamine and glutamate concentrations may be analogous to alterations in PMN glycolysis that cause maleficent intracellular conditions adversely affecting PMN function (Castell et al., 1994; Fürst et al., 1987; Newsholme et al., 1985). Interestingly, changes in aspartate concentration may also have important consequences for PMN respiratory energy sources, because aspartate, just like glutamate and α-ketoglutarate, enters the tricarboxylic acid cycle too (Newsholme et al., 1987). Past findings have shown that a combination of PMN free aspartate, glycine, ornithine, arginine but also glutamate and glutamine concentrations were highly predictive regarding the levels of PMN energy charge (Metcoff et al., 1983). Moreover, midazolam decreased intracellular arginine, ornithine and citrulline levels which favors the hypothesis that increases in PMN free arginine concentrations is followed by a decreased arginine conversion into important arginine-dependent amino acid metabolites and especially ornithine, which in undisturbed leukocyte metabolism is rapidly converted from arginine by argininase and may share the immunostimulatory and secretagogue effects of arginine (Reyero and Dorner, 1975; Evoy et al., 1998; Kriegbaum et al., 1987). This may also have essential consequences for PMN function because various findings suggest that changes in arginine turnover may underlie a significant cellular immune depression (Angele et al., 1999; Brittenden et al., 1994). For example Evoy and colleagues (Evoy et al., 1998) reported that supplementation of arginine in tumor patients stimulated lymphocyte function. Midazolam also altered the PMN free branched chain amino- (leucine, isoleucine, valine) and α-keto acid (α-ketoisovaleriate, α-ketoisocapronate and α-keto-β-methylvaleriate) as well as the threonine (α-ketobutyrate) and methionine pools which in leucocytes may play essential roles as regulators in PMN protein turnover (Metcoff et al., 1989). Metcoff (1986) who described the feasibility of using circulating PML for quantifying protein synthesis in adults, observed that cellular protein synthesis can be predicted by the intracellular levels of ''key amino acids'' such as branched chain amino acids, threonine, methionine and histidine. Admittedly, a complex phenomenon such as protein synthesis depends on the simultaneous interactive effects achieved by the biological variable in the system that regulates it, and free amino acids represent only one of these. However, it is well known that impaired amino acid metabolism (deficiencies or excesses) can limit the rate of protein synthesis, despite all other factors remaining equal (Metcoff et al., 1987, 1988). Intracellular amino acids profiles in particular appear to be better predictors than extracellular levels because all amino acids contributing to protein biosynthesis pass mainly through the dynamic free intracellular pool (Metcoff, 1986). Amino acids act in PML and other immune cells as a substrate for acute phase proteins and immunoglobulin synthesis, and in combination with polyamines in cellular replication (Castell et al., 1997; Walters et al., 1992). Alterations in the intracellular pattern of amino acids can certainly result in limitations of important PML functions such as the respiratory burst. Hazen and co-workers (Hazen et al., 1996, 1998a, b) indeed showed that active protein biosynthesis is required to sustain the ability of human neutrophils to generate oxidants. Regarding PMN immune functions, midazolam significantly decreased both superoxide anion and hydrogen peroxide generation as well as myeloperoxidase activity, suggesting an undisputable reduction in PMN function brought about by midazolam treatment. Our findings confirm former investigations in which midazolam impaired PMN immune functions (Davidson et al., 1995; Nishina et al., 1998; Tian et al., 1994; Weiss et al., 1993; Krumholz, 1995a, b). Moreover, changes in PMN immune functions documented in our study occurred in a manner closely associated (dosage and incubation period) with changes in free PMN amino- and α-keto acid concentrations.

As a possible mechanism regarding the signal transmission of anesthetic-induced cellular metabolic changes, peripheral benzodiazepine receptors could be identified here in a manner consistent with earlier immunological findings. PMN cells on their granulocytic plasma and mitochondrial membranes possess benzodiazepine receptors of the peripheral type, although the precise physiological tasks associated with them have not yet been adequately elucidated (a potential pathophysiological association with granulocytic apoptotic mechanisms has recently been talked about) (Zavala et al., 1992; Carayon et al., 1996; Stoebner et al., 2001). Up to now two isoforms of this receptor have been described, but only the benzodiazepine receptor of the so-called ''peripheral type'' is expressed in large numbers on granulocytic membranes (compared to lymphocytes or monocytes) (Pawlikowski et al., 1988; Lesnichuk et al., 1998). Those

of the ''central type'' are found mainly on brain cell membranes (i.e. glia cells) (Caldiroli et al., 1997a,b, 1998, 1999). As the present findings have confirmed, application of a receptor agonist for ''peripheral'' benzodiazepine receptors (for example Ro-4864) can mimic the midazolam-dependent influence on intragranulocytic amino- and α-keto acid pools as well as the immune functional parameters mentioned here. The combined application of midazolam and the benzodiazepine receptor agonist leads to midazolam dose-dependent additive effects. Conversely, the observed changes at therapeutic midazolam concentrations can be completely reversed by application of specific receptor antagonists for ''peripheral'' benzodiazepine receptors (PK-11195), while application of a specific antagonist for a so-called ''central'' benzodiazepine receptor (flumazenil = Ro 15-1788) has no effect. A partially antagonizing influence of PK-11195, however, could also be verified at high midazolam doses. Intrinsic effects of the benzodiazepine receptor antagonists employed could not be observed, an observation consistent with those from other research groups. These results confirm and complete earlier studies on this subject. Laghi et al. (1987), Consentino et al. (2000), Marino et al. (2001) as well as Finnerty et al. (1991) were able to show that the stimulation of peripheral benzodiazepine receptors by diazepam and Ro-4864 leads to calcium-dependent alterations of granulocyte chemiluminescence, superoxide anion production, phagocytosis and migratability, that could be reversed partially or even completely by application of specific antagonists for peripheral benzodiazepine receptors (i.e. PK-11195). Moreover, concerning the influence of flumazenil (Ro 15-1788) or other agonists or antagonists for central benzodiazepine receptors on PMN function, our findings confirm earlier studies which also showed that the application of flumazenil (Ro 15-1788) or other agonists or antagonists for central benzodiazepine receptors did not lead to any additive or antagonizing effects (Ramseier et al., 1993; Lesnichuk et al., 1998).

Overall, the results up until now confirm that intravenously applicable anesthetics, such as midazolam, can induce significant dose- as well as exposure-time-dependent changes in the free intracellular amino- and α-keto-acid pools, and parallel to that changes in important granulocytic immune functional parameters. As a potential mechanism of signal transduction for anesthetic-induced changes in cellular metabolism, an influence of peripheral benzodiazepine receptors could be identified for the first time. Benzodiazepine receptor-mediated effects consistent with earlier immune-functional results were also revealed in the granulocytic immune functional parameters. The influence on cellular glutamine-, arginine-, ornithine- and taurine-dependent metabolic processes as well as the influencing of intracellular amino acid conversion to their metabolically active α-ketoacids must be considered as highly significant in a metabolic sense. Although this study was not able to confirm any direct relationship between free amino- and α-keto acid and PMN immune functions or whether alterations in PMN amino- and α-keto acid profiles reflect direct changes of specific PMN intracellular pathways, there is significant relevance to the pharmacological regimens which enhance the supply of midazolam in whole blood. With regards to our results, we suggest that it is partially through its effect on PMN ''dynamic free amino- and α-keto acid pools'', which may be one of the determinants in cell nutrition that positively or adversely influences PMN function, that maleficent pharmacological stress (for example induced by midazolam) may have an unintentional influence on PMN immune functions. Clinically, these findings are of great importance since the combination of medication-induced inhibition/blocking of cellular amino acid metabolic processes with the loss of amino and α-ketoacid substrate homoeostasis (important for undisrupted cellular function) can induce further pathological modifications of granulocytic integrity and function, especially amongst patients with already-existing and serious disease-conditioned alterations of intracellular metabolism or systemic/intracellular substrate content. Additional (also clinical) research is required to confirm the diverse metabolic and physiological effects of medication-based therapeutic procedures on intragranulocytic amino- and α-keto acid metabolism.

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