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Involvement of Adenosine in the Antiinflammatory Action of Ketamine

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Background: **Ketamine is an anesthetic drug. Subanesthetic doses of ketamine have been shown to reduce interleukin-6 concentrations after surgery and to reduce mortality and the production of tumor necrosis factor α and interleukin 6 in septic animals. Similarly, adenosine was shown to reduce tumor necrosis factor α and mortality of septic animals. The aim of this study was to determine whether adenosine mediates the antiinflammatory effects of ketamine.**

Methods: **Sepsis was induced in mice by lipopolysaccharide or *Escherichia coli* inoculation. Leukocyte recruitment and cytokine concentrations were used as inflammation markers. Adenosine concentrations were assayed by high-performance liquid chromatography, and the involvement of adenosine in the effects of ketamine was demonstrated by adenosine receptor agonists and antagonists.**

Results: **Ketamine markedly reduced mortality from sepsis, leukocyte recruitment, and tumor necrosis factor-α and interleukin-6 concentrations. Ketamine administration in mice and rats was associated with a surge at 20–35 min of adenosine in serum (up to 5 μM) and peritoneal fluid. The adenosine A2A receptor agonist CGS-21680 mimicked the effect of ketamine in peritonitis, whereas the A2A receptor antagonists DMPX and ZM 241385 blocked its antiinflammatory effects. In contrast, A1 and A3 receptor antagonists had no effect. ZM 241385 reversed the beneficial effect of ketamine on survival from bacterial sepsis.**

Conclusions: **The current data suggest that the sepsis-protective antiinflammatory effects of ketamine are mediated by the release of adenosine acting through the A2A receptor.**

KETAMINE, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, is commonly used as an intravenous or intramuscular anesthetic[1] in patients with septicemia or trauma.[2] Various studies have shown that in addition to its anesthetic activity, ketamine has an antiinflammatory effect. For example, in clinical studies, ketamine given before surgical intervention, such as hysterectomy and coronary artery bypass grafting, significantly reduced interleukin (IL)-6 serum concentrations,[3] and in coronary bypass patients, when added in small doses to opioids during general anesthesia, ketamine down-regulated the production of superoxide anion by neutrophils.[4]

The antiinflammatory effect of ketamine has also been demonstrated in various animal models, where it was observed that ketamine strongly suppressed the production of tumor necrosis factor α (TNF-α) and IL-6 after stimulation of lipopolysaccharide.[5–7] Ketamine also significantly inhibited hypotension and metabolic acidosis in rats and minimized lethality in carrageenan-sensitized mice injected with endotoxin.[5,8,9] In clinical and experimental animal studies, the antiinflammatory effect of ketamine was obtained in subanesthetic doses of the drug.

Several recent *in vitro* studies have demonstrated that ketamine suppressed the production and release of various cytokines. For example, ketamine inhibited the leukocyte production of TNF-α, IL-6, IL-8, and nitric oxide and significantly suppressed oxygen radical generation of isolated human neutrophils.[10–12] However, in all these studies, the action of ketamine was detected only with concentrations that were 10- to 1,000-fold higher than therapeutic plasma concentrations. In a recent publication, we demonstrated that at this high concentration, ketamine exerts nonspecific cytostatic effects, arrest of cell proliferation, and blockade of cytokine production.[13] In view of all these diverse findings, no clear definitive mechanism for the antiinflammatory action of ketamine has been suggested.

Because in a previous *in vitro* study we had found that therapeutic concentrations of ketamine had no effect on cytokine secretion from leukocytes,[13] we hypothesized that an antiinflammatory mediator, such as adenosine, is induced *in vivo* after ketamine administration. Adenosine seemed to be a likely candidate for the following reasons: (1) It is a potent antiinflammatory agent that has previously been shown to decrease mortality caused by sepsis[14]; (2) it is released in the periphery by nerves during pain and inflammation[14]; and (3) in a study using an air pouch model, the antiinflammatory activity of the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP-5) was demonstrated and proven to be mediated by adenosine release.[15]

Adenosine is a nucleoside formed in the body by the enzymatic breakdown of adenosine triphosphate. Adenosine is not a typical hormone or neurotransmitter, but rather a neuromodulator in the central and peripheral nervous systems. Tissue damage and inflammation are accompanied by an accumulation of extracellular aden-

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osine due to its release from nonimmune and immune cells.[16] Adenosine interacts with at least four different G-coupled receptors: A1, A2A, A2B, and A3.

The aim of the current study was to test our hypothesis that adenosine is induced after ketamine administration and mediates its antiinflammatory activity. To investigate this issue, we measured adenosine systemic concentrations after ketamine administration and tested the ability of the adenosine receptor antagonists to blunt the antiinflammatory effect of ketamine.

Materials and Methods

Experimental Protocol

To understand the mechanism of the protective effect of ketamine, we used two models of septic shock and followed the survival of treated animals. Initially, we used endotoxin (lipopolysaccharide)–induced sepsis to define the optimal dose and treatment time of ketamine. In the second model, sepsis was induced by intraperitoneal inoculation of lethal dose of *Escherichia coli*, a model more relevant to the clinical practice. The latter model, with sublethal doses of bacteria, was used to study the antiinflammatory effect of ketamine on leukocyte recruitment and cytokine production. We used sublethal doses of bacteria to reduce mortality at harvest time. We hypothesized that ketamine induces adenosine. Therefore, we defined adenosine concentrations in sera and peritoneal fluid after ketamine administration. For this purpose, blood samples were taken from rats at several time points after injection of ketamine. Rats were used for this experiment because we needed a large volume of blood for the multiple sampling. To rule out the induction of adenosine by hemorrhage and to confirm the induction of adenosine in our experimental model, we tested adenosine concentrations in mice. To overcome blood volume limitation, samples were taken once from separate groups of mice for each time point. To prove that adenosine is involved in the antiinflammatory effect of ketamine, we mimicked its effect on leukocyte recruitment and inhibition of cytokine production by an adenosine receptor agonist and blocked ketamine effects by adenosine receptor antagonists. Finally, we blocked the sepsis-protective effect of ketamine by an adenosine receptor antagonist in the bacterial septic model.

Mice, Bacterial Strains, and Drugs

CD1 mice and Sprague-Dawley rats were bred and maintained in the animal laboratory of the Soroka Medical Center (Beer-Sheva, Israel). Experiments were done with the permission of the Ben-Gurion University of the Negev Committee for Ethical Care and Use of Animals in Experiments (Beer-Sheva, Israel). We used female mice aged 10–12 weeks.

Escherichia coli were grown in Luria-Bertani broth (Conda Laboratories, Madrid, Spain) and harvested during the log phase. Aliquots were stored frozen in LB broth containing 30% glycerol.

Adenosine (Adenocor) was purchased from Sanofi Winthrop (Auckland, New Zealand). Adenosine receptor agonists and antagonists were purchased from Sigma (Rehovot, Israel). These included CGS-21680, which is an agonist of the A2 receptor (A2R); A2R antagonist 1,3-dimethyl-7-propargylxanthine (DMPX); A1 receptor (A1R) antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX); and A3 receptor (A3R) antagonist MRS-1220 (MRS). ZM 241385, an A2A receptor (A2AR) antagonist, was purchased from Tocris (Bristol, United Kingdom). The NMDA receptor antagonist ketamine was purchased from Parke Davis (Ketalar, Hampshire, United Kingdom).

Model of Sepsis, Peritonitis, and Drug Injection

Sepsis was induced in CD1 mice by intraperitoneal injection of a lethal dose of lipopolysaccharide (150 mg/kg, *E. coli* 055:B5; Sigma) or by inoculation with aliquots of *E. coli* (3×10^7 colony-forming units). Peritonitis was induced in CD1 mice by inoculation of a sublethal dose of *E. coli* (1.5×10^7 colony-forming units). At the end of each experiment, mice were killed by neck dislocation. Peritoneal leukocytes were harvested at indicated time points after inoculation by peritoneal lavage with phosphate-buffered saline containing 2% bovine serum albumin and 5 mM EDTA. Cells were washed once, and then total leukocyte counts were determined. Ketamine was injected subcutaneously; adenosine agonists and antagonists were injected intraperitoneally. Blood for bacteriologic culture was collected in culture vials (BD Bactec plus aerobic/F; Becton Dickinson, Spark, MA) and analyzed by the Clinical Bacteriologic Laboratory at the Soroka University Medical Center.

For continuous kinetic study, female Sprague-Dawley rats (weighing 315–345 g) were used. Rats were treated with ketamine (10 mg/kg) or saline at time 0. At time 0 and at 5- or 10-min intervals, blood was drawn by heart puncture for determination of plasma adenosine concentrations.

For kinetics experiments, CD1 mice were injected subcutaneously with saline or with ketamine (10 mg/kg), with or without an intraperitoneal lethal dose of *E. coli*. Groups of three mice were bled by heart puncture at 5-min intervals for plasma adenosine concentrations.

Adenosine receptor agonist and antagonists were injected intraperitoneally. Animals were treated with the A2R agonist CGS-21680 (2 mg/kg) and with the adenosine-receptor antagonists DMPX (10 mg/kg), ZM 241385 (1 mg/kg), DPCPX (10 mg/kg), and MRS (0.2 mg/kg) 1 h before inoculation.

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Lavage, Leukocyte Counting, and Cytokine Detection

At different time points after *E. coli* inoculation, animals were killed and peritoneal lavage was performed. The lavage leukocytes were counted after trypan blue staining. After centrifugation at 400*g* for 10 min, the cell-free supernatants were removed and kept frozen at −20°C until analysis. TNF-α and IL-6 concentrations were measured by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). Cell counts and enzyme-linked immunosorbent assay were performed blindly on coded samples.

Serum Cytokine Detection

Peritonitis was induced by inoculation of *E. coli*. At different time points, animals were killed and a 1-ml syringe flushed with heparin was used to draw a blood sample from their hearts. The samples were stored on ice before centrifugation at 1,000*g* and 4°C for 10 min. The cell-free supernatants were collected and frozen at −20°C for future analysis. TNF-α and IL-6 serum concentrations were quantified by enzyme-linked immunosorbent assay.

Adenosine Determination

For adenosine determination in mice peritoneal lavage, 900 μl lavage fluid was placed in a polystyrene tube containing 100 μl stop solution (2.3 μM erytro-9(2-hydroxy non-3-yl)-adenine and 20 μM dipyridamole) designed to inhibit the metabolism and cellular uptake of adenosine.[17] For plasma adenosine determination, a 1-ml syringe containing a small amount of heparin and 50 μl stop solution was used to draw a 0.5-ml blood sample.

The method for measuring adenosine in deproteinated fluid was described previously.[18] Briefly, cell-free fluid was combined with methanol in a ratio of 2:3 and heated at 100°C for 3 min to extract proteins. After centrifugation at 13,000*g* for 5 min, the supernatant was removed, dried with a centrifuge-vacuum concentrator, and reconstituted with 500 μl water. Then, the solution was mixed with 20 μl acetate buffer, 2.0 M, pH 4.5, and 10 μl (50% in water) chloracetaldehyde (Sigma), heated at 80°C for 2 h. The samples were protected from light during and after heating. After cooling to room temperature, the samples were briefly centrifuged and a portion (typically 50 μl) was directly injected onto a C18 high-performance liquid chromatography (HPLC) column for analysis (see HPLC Analysis). Samples could be stored at 4°C before HPLC analysis for at least 48 h without noticeable degradation. Adenosine determination was performed blindly on coded samples.

HPLC Analysis

The HPLC system used in this study was equipped with a Quaternary pump, an Agilent 1100 autosampler, and an Agilent 1100 fluorescence detector. Samples were chromatographed on a Kromasil KR100-5C18 column (5 mm, 150 × 4.60 mm; cat. No. E8285; Agilent, Edison, NJ). For determination of 1-N^6-ethenoadenosine, samples were eluted with 20% methanol in water in an isocratic mode with a flow rate of 1,500 ml/min. The fluorescent product 1-N^6-ethenoadenosine was eluted at 2.4 min and was detected with an Agilent 1100 fluorescence detector set at an excitation wavelength of 270 nm and an emission wavelength of 411 nm. Adenosine concentrations were calculated by measuring peak areas and comparing these with peaks produced by adenosine standards of known concentrations.

Flow Cytometry Analysis

For flow cytometry analysis, peritoneal leukocytes were harvested and rinsed with phosphate-buffered saline. Then, 1.5×10^6 cells were stained in 100 μl phosphate-buffered saline containing 1% bovine serum albumin and 0.05% sodium azide for 1 h on ice with a labeled leukocyte marker. The following monoclonal antibodies were used: fluorescein isothiocyanate–conjugated rat anti-mouse F4/80 monoclonal antibody (clone CI:A3-1; Serotec, Raleigh, NC); FITC-conjugated rat anti-mouse Ly-6G (GR-1) monoclonal antibody (clone RB6-8C5; Pharmingen, San Diego, CA); biotin-conjugated hamster anti-mouse CD3ϵ monoclonal antibody (clone 500A2; Pharmingen); and R-pycoerythrin–conjugated streptavidin (Jackson, West Grove, PE).

After staining, cells were washed and fixed in 0.2% paraformaldehyde–phosphate-buffered saline. Antibodies were diluted to recommended concentrations according to the manufacturer's instructions. Nonspecific binding of antibodies was adjusted with cells labeled with matching isotype control antibodies. At least 10,000 cells were analyzed per sample. Analyses were conducted by flow cytometry (FACS Calibur; Becton Dickinson, Mountain View, CA). Fluorescence data were analyzed by the Cell Quest Program (Becton Dickinson).

Statistical Analysis

Survival rates of ketamine-treated and control rats were compared using a one-tailed log-rank test, and one-way analysis of variance (followed by Tukey test) was used to compare among the various treatment groups. *P* values less than 0.05 were considered significant.

Results

To determine the effect of ketamine on survival, three groups of mice were injected with a lethal dose of lipopolysaccharide. As shown in figures 1 and 2, a dose of 10 mg/kg given before lipopolysaccharide administration increased the survival rates of mice to 86% compared with 8% in untreated mice. However, when injected 1 h (not shown) or 2 h after lipopolysaccharide

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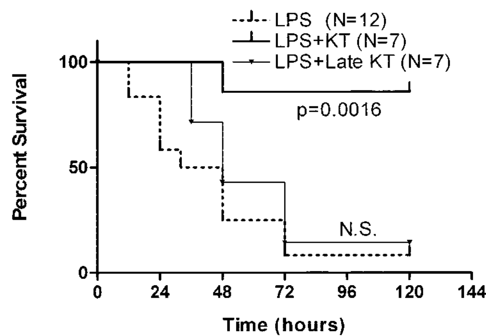

Fig. 1. Effect of ketamine treatment on survival from lipopolysaccharide-induced sepsis. Sepsis was induced in CD1 mice by intraperitoneal injection of lethal doses of lipopolysaccharide (LPS, 150 mg/kg). Mice were treated by subcutaneous injection of saline in the control group (LPS) or with ketamine (10 mg/kg) at the time of lipopolysaccharide injection (LPS + KT) or 2 h after lipopolysaccharide injection (LPS + Late KT). Mortality was followed up to 5 days. Treated groups were compared with the LPS group. NS = no significant difference. The figure depicts a representative experiment out of three performed.

administration (fig. 1), the same dose did not improve survival rates as compared with untreated animals. In experiments aimed at determining the optimal dosage of ketamine (fig. 2), we found a positive dose response to 0.5–10 mg/kg ketamine (fig. 2). Therefore, we used 10 mg/kg ketamine for the rest of the experiments.

To investigate whether the antiinflammatory properties of ketamine are mediated by release of adenosine, we measured adenosine concentrations in biologic fluids. As shown in figure 3A, the adenosine concentration peaked at 2.4 min in an HPLC analytical method developed here. Pretreatment of the same sample with adenosine deaminase completely eliminated the adenosine peak (fig. 3B), indicating the specificity of the assay. Integration of adenosine standard gave a linear dose response from 0.01 to 5 μM (not shown). To assess the direct effect of ketamine on blood adenosine concentration, one rat was injected with ketamine and a control rat was injected with saline; blood samples were taken from them at 5-min intervals (fig. 4A). In contrast to the control saline-injected rat in the ketamine-injected animal, a sharp peak of adenosine reaching 5 μM was seen 25–30 min after injection. To ascertain that the increase of adenosine in the ketamine-treated animals was indeed due to ketamine treatment and not to the stress of massive bleeding, we killed mice at 5-min intervals and analyzed their blood for adenosine concentration (fig. 4B). Similar to our findings in rats, adenosine serum concentrations in mice peaked at 25 min after ketamine injection and reached a concentration of approximately 0.4 μM. In the experiments with mice, we were able to see a significant increase of adenosine serum concentrations as early as 15 min after ketamine administration. The same increase in adenosine concentration was observed when mice were inoculated with a lethal dose of

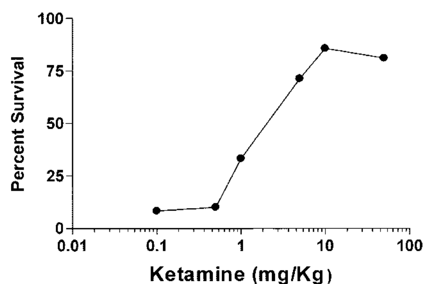

Fig. 2. Dose-dependent effect of ketamine on mice survival. Sepsis was induced in CD1 mice by intraperitoneal injection of lethal doses of lipopolysaccharide (150 mg/kg). Mice were treated subcutaneously with increasing doses of ketamine at time of lipopolysaccharide injection. The mortality rate was evaluated at 96 h. n = 12 for the ketamine concentration point of 10 mg/kg, and n = 7 for other points.

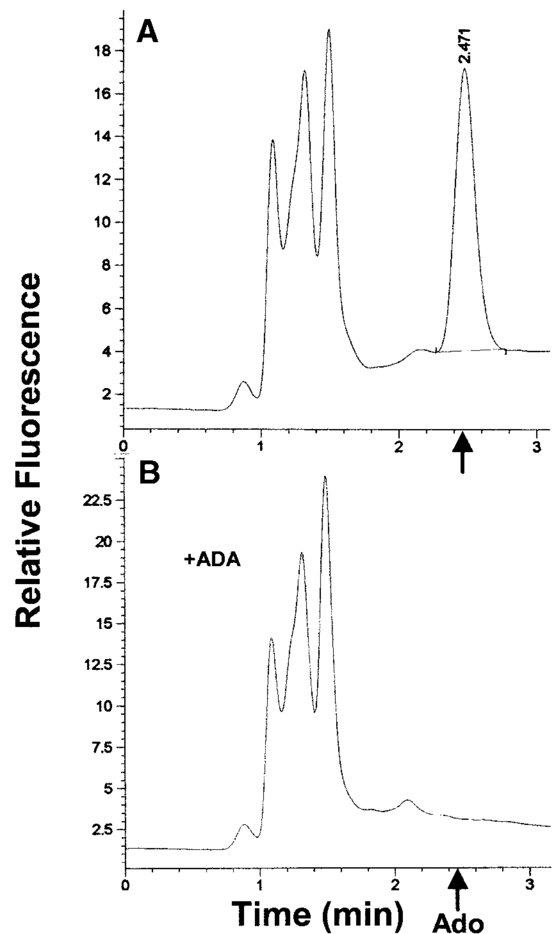

Fig. 3. Specificity of high-performance liquid chromatography (HPLC) chromatograms of mice plasma for adenosine determination. For evaluation of adenosine concentrations, blood was taken in the presence of heparin, plasma was separated and deproteinated, and adenosine was derivatized to a fluorescent metabolite, 1-*N*[6]-ethenoadenosine. Analysis was performed using the C18 HPLC column to give the chromatographs shown. The *arrows* indicate the time point of the adenosine standard peak. (*A*) HPLC of plasma sample, 25 min after ketamine administration. (*B*) HPLC of the same sample pretreated with adenosine deaminase (ADA, 5 U/ml) for 20 min at 37°C. The figure depicts a representative HPLC analysis. Treatment with adenosine deaminase was performed in three independent experiments with similar results.

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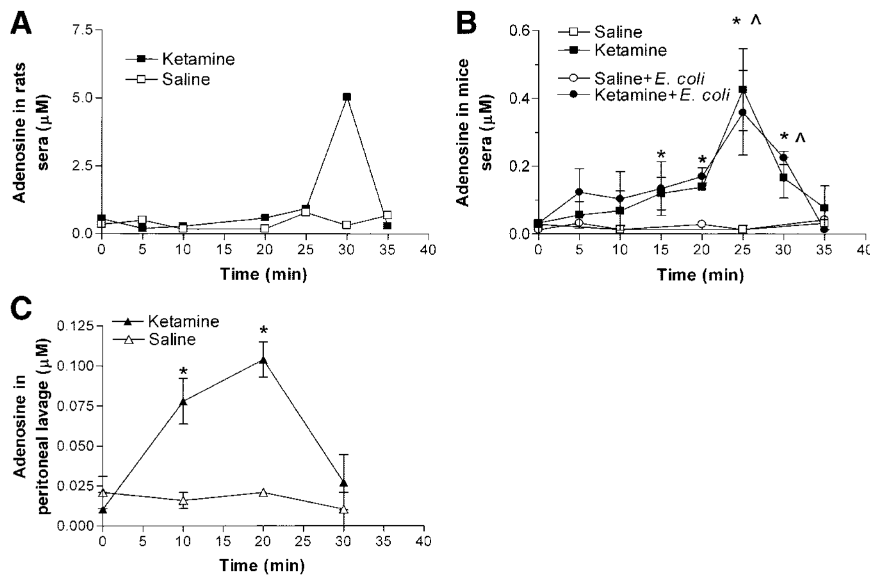

Fig. 4. Kinetics of adenosine induction after ketamine administration. (*A*) Saline or ketamine (10 mg/kg) was injected subcutaneous to a rat. At each time point, the same animal was bled by heart puncture for adenosine determination. (*B*) Saline or ketamine (10 mg/kg) was injected subcutaneously with or without an intraperitoneal inoculation of a lethal dose of *E. coli* (3×10^7 colony-forming units) to CD1 mice or injected with saline. After treatment CD1 mice were bled by heart puncture and killed (n = 3 for each time point), (*C*) saline or ketamine (10 mg/kg) was injected subcutaneously. After treatment at the indicated time points, mice were killed, and peritoneal lavage was performed (n = 3 for each time point). Adenosine concentrations in sera and lavage fluids were determined by high-performance liquid chromatography as described in figure 3. *$P < 0.05$ for ketamine treatment compared with time 0. $P < 0.05$ for ketamine plus *E. coli* treatment compared with time 0. Each graph in the figure depicts a representative experiment out of three performed.

E. coli in addition to ketamine administration (fig. 4B). To test whether a similar increase in adenosine concentration after ketamine administration occurs in the peritoneal cavity, we measured adenosine concentration in peritoneal lavage after ketamine administration. As shown in figure 4C, similar to the increase of adenosine in mice blood, we found a significant increase in adenosine concentrations in the peritoneum starting from 10 min, and then a decline at 30 min. To reduce distress of the experimental animals caused from the long mortality experiments, we continued the study in a shorter and less painful model of bacterial peritonitis. For this reason, two groups of mice were inoculated intraperitoneally with a sublethal dose of *E. coli* and treated subcutaneously with saline or ketamine (10 mg/kg). Cell exudates were collected at various time points by peritoneal lavage. As shown in figure 5, a half lethal dose of *E. coli* inoculation to the peritoneum induced massive leukocyte recruitment, which peaked at 12 h and slowly

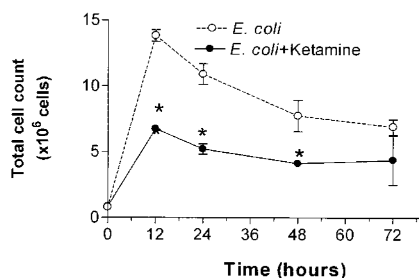

Fig. 5. Effect of ketamine on leukocyte recruitment during peritonitis. CD1 mice (n = 6 per time point) were inoculated intraperitoneally with a sublethal dose of *E. coli* (1.5×10^7 colony-forming units) and treated subcutaneously with saline or ketamine (10 mg/kg). Cell exudates were collected by peritoneal lavage. Results are presented as mean ± SE. *$P < 0.05$ for ketamine treatment compared with saline treatment for the same time point. The figure depicts a representative experiment out of three performed.

declined. At every time point tested, after ketamine administration, leukocyte recruitment was reduced by approximately 50% compared with saline-treated animals. Ketamine had no effect on the leukocyte profile in the peritoneal lavage as analyzed by flow cytometry using specific markers (data not shown).

To support our finding that the increase in adenosine concentrations after ketamine administration is responsible for the antiinflammatory activity of ketamine, we examined the effects of adenosine receptor antagonists and agonists in a mouse peritonitis model. Mice were injected with saline or inoculated with a sublethal dose of *E. coli*. Inoculated mice were pretreated with saline; with ketamine; with an A2R antagonist, DMPX; with DMPX and ketamine; with an A2AR agonist, CGS-21680; with a specific A2AR antagonist; with ZM 241385; or with ketamine and ZM 241385. Similar to the effect of ketamine, the adenosine A2AR agonist CGS-21680 significantly reduced peritoneal leukocyte recruitment after *E. coli* inoculation. The A2R antagonist DMPX and the specific A2AR antagonist ZM 241385, however, effectively reversed the inhibitory effect of ketamine (fig. 6). We then investigated the effect of ketamine and the A2R agonist and antagonist on TNF-α and IL-6 concentrations. As shown in figure 7, ketamine and the A2AR agonist CGS-21680 reduced TNF-α and IL-6 concentrations in the serum (figs. 7A and B) and peritoneal fluid (figs. 7C and D) of the mice with peritonitis. The effect of ketamine and CGS-21680 reached statistical significance except to the inhibitory effect of CGS-21680 on TNF-α concentration in the peritoneum (fig. 7C). Like the effect on leukocyte recruitment, the inhibitory effect of ketamine on TNF-α and IL-6 concentrations in blood and peritoneal fluids was blocked by DMPX (figs. 7A–D). In addition, ZM 241385 effectively reversed the inhibitory effect of ketamine on TNF-α and IL-6 secretion (figs.

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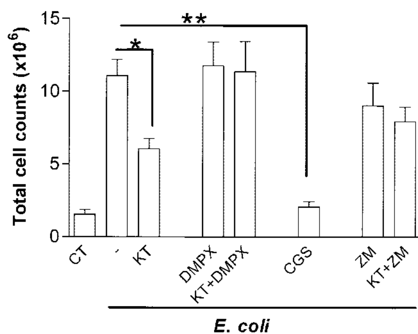

Fig. 6. Ketamine-induced inhibition of leukocyte recruitment is reverted by adenosine receptor antagonists. CD1 mice (n = 6 per treatment) were injected with saline (CT) or inoculated with a sublethal dose of *E. coli*. One hour before inoculation, mice were pretreated as indicated with saline (−); with an A2 receptor antagonist, DMPX (10 mg/kg); with an A2A receptor agonist, CGS-21680 (CGS, 2 mg/kg); and with a specific A2A receptor antagonist, ZM 241385 (ZM, 1 mg/kg). At the time of inoculation, mice were treated with saline (not indicated) or ketamine (KT, 10 mg/kg). Cell exudates were collected at 24 h by peritoneal lavage and counted. Results are presented as mean ± SE. * $P < 0.05$, ** $P < 0.01$. The figure depicts a representative experiment out of three performed.

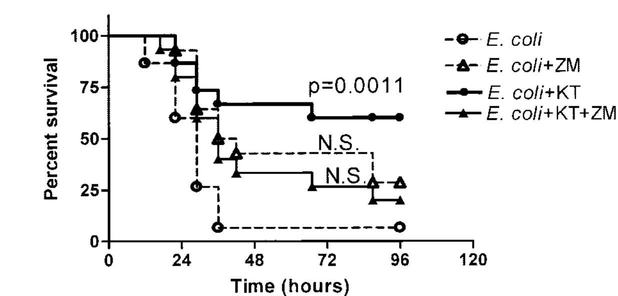

Fig. 8. Effect of adenosine A2A receptor antagonist on ketamine-induced survival from gram-negative bacterial sepsis. Sepsis was induced in CD1 mice (n = 15 for each treatment) by intraperitoneal inoculation of a lethal dose of *E. coli* (3×10^7 colony-forming units). One hour before inoculation, mice were pretreated as indicated with saline (*E. coli* and *E. coli* + KT groups) or with ZM 241385 (1 mg/kg, *E. coli* + ZM and *E. coli* + KT + ZM groups). At the time of inoculation, mice were treated subcutaneously with saline or with ketamine (KT, 10 mg/kg), and mortality was followed up to 4 days. Treated groups were compared with the *E. coli* group. NS = no significant difference.

7A–D). In contrast to A2R antagonists, the A1 and A3 adenosine receptor antagonists DPCPX and MRS had no effect on ketamine inhibition (figs. 7A and B). Similar to the effect on survival, late ketamine injection (2 h after inoculation) did not significantly reduce cell recruitment or TNF-α and IL-6 concentrations in the serum (data not shown). DPCPX, DMPX, ZM 241385, and MRS without ketamine had no effect on serum and peritoneal TNF-α and IL-6 concentrations induced by *E. coli* inoculation (data not shown). Finally, to prove that the beneficial effect of ketamine on survival is mediated by adenosine and is not purely circumstantial, we inoculated animals with a lethal dose of *E. coli* and tested the effect of the A2A receptor antagonist ZM 241385. Similar to the beneficial effect of ketamine treatment in the lipopolysaccharide studies, we found that ketamine significantly increased survival rate of *E. coli* inoculated mice to 60%, compared with untreated animals, which had only a 6.7% survival rate (fig. 8). Similar to the effect on leukocyte recruitment and cytokine secretion, ZM 241385 blocked the beneficial effect of ketamine and reduced survival to 20%. The survival rate of mice inoculated and

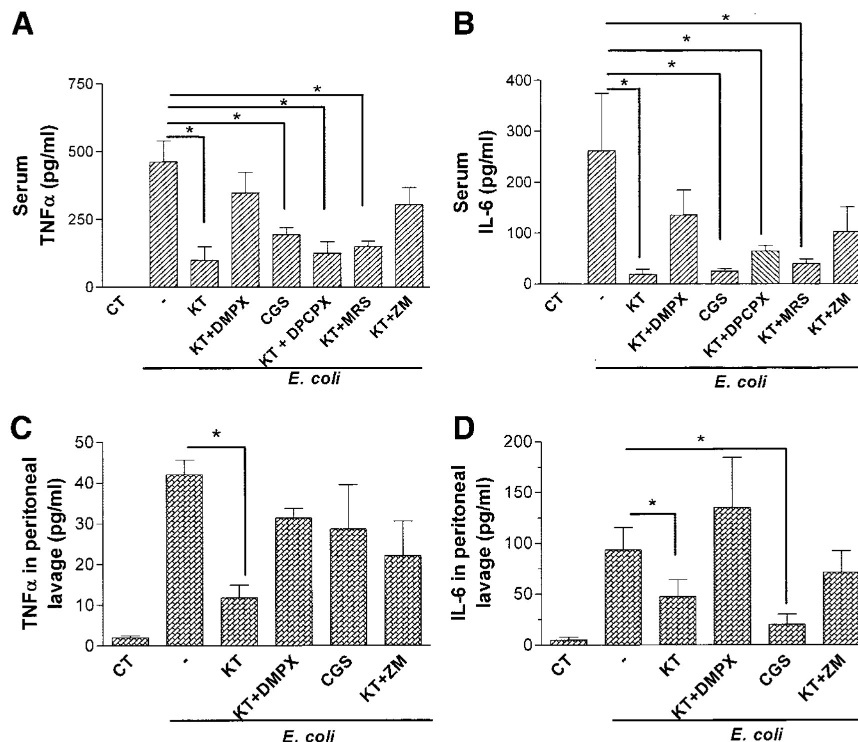

Fig. 7. Ketamine-induced inhibition of local and system cytokine concentrations is reverted by an adenosine receptor antagonist. CD1 mice (n = 4 per treatment) were injected intraperitoneally with saline (CT) or inoculated with a sublethal dose of *E. coli*. One hour before inoculation, mice were pretreated as indicated with saline (−); with an A2 receptor antagonist, DMPX (10 mg/kg); with an A2A receptor agonist, CGS-21680 (CGS, 2 mg/kg); with an A1 receptor antagonist, DPCPX (10 mg/kg); with an A3 receptor antagonist, MRS (0.2 mg/kg); and with an A2A receptor antagonist, ZM 241385 (ZM, 1 mg/kg). At the time of inoculation, mice were treated with saline (not indicated) or ketamine (KT, 10 mg/kg). Animals were bled at 3 h for determination of sera tumor necrosis factor α (TNF-α; *A*) and at 6 h for determination of sera interleukin 6 (IL-6; *B*). For analysis of cytokines in peritoneum, lavage was performed at 24 h, and the cell-free exudates were collected and subsequently analyzed for determination of TNF-α (*C*) and IL-6 (*D*). TNF-α and IL-6 were analyzed by specific enzyme-linked immunosorbent assay. Results are presented as mean ± SE. * $P < 0.05$. The figure depicts a representative experiment out of three performed.

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treated with ZM 241385 was not significantly different compared with the untreated control group inoculated with *E. coli*. Blood bacteriologic culture indicated that all four groups had development of bacteremia at 24 h, and their peritoneal lavage was positive for *E. coli* (data not shown).

Discussion

Our objective was to elucidate the mechanism of the antiinflammatory action of ketamine. We found that reduced mortality of mice treated with ketamine is dose related, with a maximal effect at the subanesthetic dose of 10 mg/kg (the anesthetic dose of ketamine in mice and rats is approximately 100 mg/kg). In addition, we showed that ketamine is effective only when given concurrently with lipopolysaccharide but not when given 1–2 h after lipopolysaccharide administration. These findings suggest that ketamine suppresses the initial steps of the inflammatory cascade. Previous findings[5–7] and our current data show that ketamine suppresses TNF-α secretion, known to be one of the main inducers of uncontrolled inflammatory cascade observed in septic patients.[19]

Because in a previous *in vitro* study we had found that therapeutic concentrations of ketamine had no effect on cytokine secretion from leukocytes,[13] we hypothesized that an antiinflammatory mediator, such as adenosine, is induced *in vivo* after ketamine administration. In the current study, we clearly show that ketamine administration to rats and mice induced a significant increase in plasma and peritoneal adenosine concentrations, which occurs within 20–30 min after ketamine administration. The concentrations measured in sera (peak concentrations approximately 5 μM) and peritoneal concentrations (estimated concentrations 2–3 μM) lie within the physiologic range of activation of the A2AR (dissociation constant = 1.2 nM).[20] The concentrations of adenosine in the peritoneum are estimated concentrations because we have no data on the volume of intraperitoneal fluid in mice. We measured adenosine concentrations in 5 ml lavage fluid which peak at a range of 0.1 μM. We estimate that the actual peritoneal fluid volume is approximately 0.2 ml; therefore, adenosine peak concentrations are 20- to 30-fold higher (2–3 μM).

Theoretically, adenosine can be produced by any cell in the body; however, the target of ketamine is the NMDA receptor in the central nervous system, which suggests that in this case, adenosine is produced by nerve endings. Strongly supporting this possibility is the work of Bong *et al.*,[15] which demonstrated the role of adenosine in inhibition of neutrophil migration by AP-5, a glutamate NMDA receptor antagonist. Because ketamine and AP-5 are NMDA receptor blockers, it is possible that their action on this receptor in the central nervous system induces the release of adenosine by nerves in the periphery. The obtained peak of adenosine was short and sharp, a fact that can be explained by the rapid clearance of adenosine in sera (half-life = 1.5 s) by cellular uptake and/or metabolism through phosphorylation to adenosine monophosphate or through deamination to inosine.

Adenosine activities are mediated by at least four different receptors; A1 and A3 receptors are Gi protein–coupled receptors, whereas A2A and A2B receptors are Gs protein–coupled receptors that activate adenylate cyclase and cause accumulation of intracellular cyclic adenosine monophosphate. To demonstrate that adenosine mediates the antiinflammatory action of ketamine, we used different agonists and antagonists of adenosine receptors. We found that A2AR activation by the agonist CGS-21680 mimics the effect of ketamine on peritonitis. CGS-21680 significantly reduced peritoneal leukocyte recruitment as well as the increase of cytokine concentrations in blood and in the peritoneum. More direct evidence pointing to adenosine as a ketamine mediator was obtained using the A2R antagonist DMPX and the specific A2AR antagonist ZM 241385. DMPX injected before ketamine blocked the ketamine inhibition of TNF-α and IL-6 release as well as leukocyte recruitment. Similarly, ZM 241385 reversed the inhibitory effect of ketamine on leukocyte recruitment and on TNF-α and IL-6 secretion. In contrast to DMPX, A1R- and A3R-specific antagonists DPCPX and MRS did not block the effects of ketamine. The results of the survival experiments strongly support the involvement of adenosine and its A2AR in the ketamine effect. In these experiments, we demonstrated that the A2AR antagonist ZM 241385 blocked the beneficial effect of ketamine on survival from bacterial sepsis. Ketamine mimicry by the A2AR agonist and the specific blocking of ketamine effects by the A2AR antagonists therefore suggest that ketamine suppresses inflammation through the A2AR. It is important to emphasize that the antiinflammatory activity of adenosine, including inhibition of leukocyte migration and cytokine production, were shown to be predominantly mediated by the A2AR.[6] Consistent with these data, our findings suggest that A2AR is the dominant receptor of adenosine involved in ketamine activity.

Because ketamine effectively reduces cytokine secretion and mortality from sepsis only when injected immediately or shortly after lipopolysaccharide administration, we hypothesized that the ketamine-induced short adenosine surge halts the inflammatory cascade at its initial steps, perhaps during pre–TNF-α stages. Adenosine and its agonists have been shown to effectively reduce lipopolysaccharide-induced TNF-α release *in vitro* by peritoneal macrophages and by dendritic cells[21,22] and *in vivo* in mice injected with lipopolysaccharide.[23] The narrow timing of the inhibitory effect of adenosine is clearly illustrated in the work of Hasko *et*

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al.,[22] who showed that adenosine inhibits lipopolysaccharide-induced IL-12 secretion from macrophages only when given shortly after lipopolysaccharide administration. Additional evidence for the short-term action of adenosine is provided by a recent study conducted by Majumdar *et al.*,[24] who showed that TNF-α activation of nuclear factor κB could be blocked only when cells were treated with adenosine at least 30 min before TNF-α stimulation. When adenosine was given at the same time as TNF-α or later, no inhibition of nuclear factor κB was observed. The antiinflammatory effect of adenosine has been demonstrated in many other studies, which have shown that the inhibition of secretion of various chemokines and inflammatory mediators, such as TNF-α, IL-6, and IL-12, is due to activation of A2AR (reviewed in Sitkovsky[16]).

In addition to ketamine, adenosine is also considered to be the mediator of the antiinflammatory effect of methotrexate, a drug commonly used for the treatment of rheumatoid arthritis[25,26] and for the antiinflammatory drugs salicylates and sulfasalazine.[27]

Given that the antiinflammatory effect of ketamine is effective only at the initial stage of sepsis and because diagnosis is usually made at later stage, after the clinical symptoms of inflammation become manifest, patients with sepsis will probably have a limited benefit from the antiinflammatory effect of ketamine. However, administration of subanesthetic doses of ketamine might be advantageous for patients who have undergone elective surgery or other invasive procedures with a high risk of bacteriemia. It should also be noted that ketamine is beneficial in preservation of cardiovascular function of septic patients as previously described.[9]

To conclude, we propose that the sepsis-protective antiinflammatory effects of ketamine are mediated by adenosine. Ketamine administration causes release of adenosine in the periphery, and adenosine through A2AR reduces the systemic inflammatory response by inhibition of secretion of proinflammatory cytokines as well as leukocyte activation and recruitment.

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References

1. Miyasaka M, Domino EF: Neural mechanisms of ketamine-induced anesthesia. Int J Neuropharmacol 1968; 7:557–73
2. Lippmann M, Appel PL, Mok MS, Shoemaker WC: Sequential cardiorespiratory patterns of anesthetic induction with ketamine in critically ill patients. Crit Care Med 1983; 11:730–4
3. Roytblat L, Talmor D, Rachinsky M, Greemberg L, Pekar A, Appelbaum A, Gurman GM, Shapira Y, Douvdevani A: Ketamine attenuates the interleukin-6 response after cardiopulmonary bypass. Anesth Analg 1998; 87:266–71
4. Zilberstein G, Levy R, Rachinsky M, Fisher A, Greemberg L, Shapira Y, Appelbaum A, Roytblat L: Ketamine attenuates neutrophil activation after cardiopulmonary bypass. Anesth Analg 2002; 95:531–6
5. Koga K, Ogata M, Takenaka I, Matsumoto T, Shigematsu A: Ketamine suppresses tumor necrosis factor-alpha activity and mortality in carrageenan-sensitized endotoxin shock model. Circ Shock 1994; 44:160–8
6. Taniguchi T, Shibata K, Yamamoto K: Ketamine inhibits endotoxin-induced shock in rats. Anesthesiology 2001; 95:928–32
7. Taniguchi T, Takemoto Y, Kanakura H, Kidani Y, Yamamoto K: The dose-related effects of ketamine on mortality and cytokine responses to endotoxin-induced shock in rats. Anesth Analg 2003; 97:1769–72
8. Mastronardi CA, Yu WH, McCann S: Lipopolysaccharide-induced tumor necrosis factor-alpha release is controlled by the central nervous system. Neuroimmunomodulation 2001; 9:148–56
9. Van der Linden P, Gilbart E, Engelman E, Schmartz D, de Rood M, Vincent JL: Comparison of halothane, isoflurane, alfentanil, and ketamine in experimental septic shock. Anesth Analg 1990; 70:608–17
10. Shimaoka M, Iida T, Ohara A, Taenaka N, Mashimo T, Honda T, Yoshiya I: Ketamine inhibits nitric oxide production in mouse-activated macrophage-like cells. Br J Anaesth 1996; 77:238–42
11. Yu Y, Zhou Z, Xu J, Liu Z, Wang Y: Ketamine reduces NFκB activation and TNFα production in rat mononuclear cells induced by lipopolysaccharide in vitro. Ann Clin Lab Sci 2002; 32:292–8
12. Kawasaki C, Kawasaki T, Ogata M, Nandate K, Shigematsu A: Ketamine isomers suppress superantigen-induced proinflammatory cytokine production in human whole blood. Can J Anaesth 2001; 48:819–23
13. Lewis E, Rogachev B, Shaked G, Douvdevani A: The in vitro effects of ketamine at large concentrations can be attributed to a nonspecific cytostatic effect. Anesth Analg 2001; 92:927–9
14. McCallion K, Harkin DW, Gardiner KR: Role of adenosine in immunomodulation: Review of the literature. Crit Care Med 2004; 32:273–7
15. Bong GW, Rosengren S, Firestein GS: Spinal cord adenosine receptor stimulation in rats inhibits peripheral neutrophil accumulation: The role of N-methyl-D-aspartate receptors. J Clin Invest 1996; 98:2779–85
16. Sitkovsky MV: Use of the A(2A) adenosine receptor as a physiological immunosuppressor and to engineer inflammation in vivo. Biochem Pharmacol 2003; 65:493–501
17. Nithipatikom K, Mizumura T, Gross GJ: Determination of plasma adenosine by high-performance liquid chromatography with column switching and fluorometric detection. Anal Biochem 1994; 223:280–4
18. Forsythe P, McGarvey LP, Heaney LG, MacMahon J, Ennis M: Adenosine induces histamine release from human bronchoalveolar lavage mast cells. Clin Sci (Lond) 1999; 96:349–55
19. Reinhart K, Karzai W: Anti-tumor necrosis factor therapy in sepsis: Update on clinical trials and lessons learned. Crit Care Med 2001; 29:S121–5
20. Varani K, Gessi S, Merighi S, Iannotta V, Cattabriga E, Spisani S, Cadossi R, Borea PA: Effect of low frequency electromagnetic fields on A2A adenosine receptors in human neutrophils. Br J Pharmacol 2002; 136:57–66
21. Panther E, Corinti S, Idzko M, Herouy Y, Napp M, la Sala A, Girolomoni G, Norgauer J: Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T-cell stimulatory capacity of human dendritic cells. Blood 2003; 101:3985–90
22. Hasko G, Kuhel DG, Chen JF, Schwarzschild MA, Deitch EA, Mabley JG, Marton A, Szabo C: Adenosine inhibits IL-12 and TNF-α production via adenosine A2a receptor-dependent and independent mechanisms. FASEB J 2000; 14:2065–74
23. Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM, Vizi ES: Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. J Immunol 1996; 157:4634–40
24. Majumdar S, Aggarwal BB: Adenosine suppresses activation of nuclear factor-kappaB selectively induced by tumor necrosis factor in different cell types. Oncogene 2003; 22:1206–18
25. van Ede AE, Laan RF, De Abreu RA, Stegeman AB, van de Putte LB: Purine enzymes in patients with rheumatoid arthritis treated with methotrexate. Ann Rheum Dis 2002; 61:1060–4
26. Chan ES, Cronstein BN: Molecular action of methotrexate in inflammatory diseases. Arthritis Res 2002; 4:266–73
27. Cronstein BN, Montesinos MC, Weissmann G: Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NFκB. Proc Natl Acad Sci U S A 1999; 96:6377–81

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