Ketamine isomers suppress superantigen-induced proinflammatory cytokine production in human whole blood

[Les isomères de la kétamine suppriment la production de cytokine pro-inflammatoire induite par des superantigènes dans le sang complet humain]

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Purpose: To investigate the efficacy of S(+)-ketamine and R(-)-ketamine on staphylococcal enterotoxin B (SEB)-induced tumour necrosis factor (TNF)-, interleukin (IL)-6, and IL-8 production in human whole blood *in vitro*.

Methods: After Ethics Committee approval and informed consent, blood samples were obtained from ten healthy volunteers and diluted with five volumes of RPMI 1640. After adding different doses of ketamine isomers (0–1000 μM), the blood was stimulated with SEB (10 ng·mL^{-1}). After a six-hour incubation period, the plasma TNF- activity was determined by the L929 cell cytotoxic assay and IL-6 and IL-8 concentrations were measured using an enzyme-linked immunoassay.

Results: Ketamine isomers significantly suppressed SEB-induced TNF- production at concentrations exceeding 50 μM. Ketamine isomers at concentrations exceeding 100 μM also significantly suppressed SEB-induced IL-6 production. Furthermore, ketamine isomers at concentrations exceeding 500 μM significantly suppressed SEB-induced IL-8 production. There were no significant differences between the suppressive effects of S(+)-ketamine and R(-)-ketamine on SEB-induced proinflammatory cytokine production.

Conclusion: This study demonstrated that ketamine isomers suppressed SEB-induced TNF-, IL-6, and IL-8 production in human whole blood.

Objectif : *Vérifier l'efficacité de la S(+)-kétamine et de la R(-)-kétamine sur la production du facteur nécrosant des tumeurs (FNT)-a, de l'interleukine (IL)-6 et de l'IL-8, induits par l'entérotoxine B d'origine staphylococcique (EBS), dans le sang complet humain in vitro.*

Méthode : *Après avoir obtenu l'approbation du Comité d'éthique et le consentement éclairé des participants, nous avons recueilli des échantillons sanguins chez dix volontaires en santé et les avons dilué dans cinq volumes de RPMI 1640. Après l'addition de différentes doses d'isomères de kétamine (0–1000 μM), le sang a été stimulé avec l'EBS (10 ng·mL^{-1}). À la suite d'une incubation de six heures, l'activité plasmatique de TNF-a a été déterminée par le dosage de la cytotoxicité cellulaire L929, et les concentrations d'IL-6 et d'IL-8 ont été mesurées au moyen d'un dosage immuno-enzymatique.*

Résultats : *Les isomères de kétamine ont supprimé de façon significative la production du TNF-a induit par l'EBS à des concentrations dépassant 50 μM, la production d'IL-6 induite par l'EBS à des concentrations au delà de 100 μM et la production d'IL-8 induite par l'EBS à des concentrations de plus de 500 μM. Il n'y a pas eu de différence significative entre les effets suppresseurs de la S(+)-kétamine et de la R(-)-kétamine sur la production de cytokine pro-inflammatoire induite pas l'EBS.*

Conclusion : *Cette étude démontre que les isomères de kétamine suppriment la production du FNT-a, d'IL-6 et d'IL-8, induits par l'EBS, dans le sang complet humain.*

CYTOKINES play an important role in the defense against infection; however, excessive production of cytokines is associated with the development of septic shock and organ dysfunction.

Recently, the prevalence of Gram-positive bacterial pathogens as a cause of sepsis has been increasing rela-

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tively to Gram-negative pathogens. The mortality of sepsis from Gram-positive pathogens is higher than that from Gram-negative pathogens.[1–3] Enterotoxins produced by Gram-positive bacilli are potent mitogens for human T cells, monocytes, and macrophages and cause lethal toxic shock.[4] Staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) are well-known enterotoxins produced by Gram-positive bacilli. These toxins function as superantigens that activate T cells, monocytes, and macrophages by cross-linking an outside domain of the major histocompatibility complex class II molecules on antigen-presenting cells with the variable portion of the ß-chain of the T cell receptor, without the internalization and proteolysis required by conventional antigens.[5] The superantigen activity of SEs results in the synthesis of a variety of cytokines, including interleukin (IL)-1, IL-2, IL-6, IL-8, interferon (IFN)-, and tumour necrosis factor (TNF)-.[6] The massive production and release of such cytokines initiate tissue injury which can cause organ dysfunction and eventually lead to death.

Ketamine, an *iv* anesthetic, has been advocated for anesthesia of septic or severely ill patients because of its cardiovascular stimulating effects.[7,8] Ketamine increases cardiac output and systemic vascular resistance, which is thought to stimulate the sympathetic nervous system, resulting in the release of catecholamines.[9] We previously reported that ketamine suppressed lipopolysaccharide (LPS)-induced TNF- production *in vivo* and *in vitro*.[10–12] However, there are no reports on the effect of ketamine on SE-induced proinflammatory cytokine production in human whole blood.

In this study, we investigated the effects of ketamine isomers on staphylococcal enterotoxin B (SEB)-induced TNF-, IL-6, and IL-8 production in human whole blood *in vitro*.

Materials and methods

SEB was purchased from Sigma Chemical (lot. no.86H4062; St. Louis MO, USA). S(+)-ketamine and R(-)- ketamine were provided by Park-Davis GmbH (Freiburg, Germany).

After approval from our Human Investigations Committee, informed consent was obtained from ten healthy male volunteers not taking any medication. Blood samples were drawn into tubes containing heparin and diluted with five volumes of RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan).[13] One millilitre of diluted blood per well was placed into 24-well tissue culture plates (Becton Dickinson, Lincoln Park NJ, USA).

After different doses (0–1000 µM) of S(+)-ketamine or R(-)-ketamine were added to each well, whole blood was stimulated with SEB (10 $ng \cdot mL^{-1}$). Then, the blood was incubated for six hours at 37°C in a 95% air / 5% CO_2 incubator. After incubation, the blood was centrifuged at 700 *G* for ten minutes to remove blood cells. Supernatant samples were collected and stored at –80°C until assayed.

The L929 cell cytotoxic assay described previously was used to determine the plasma TNF- activity.[14] Briefly, L929 cells in RPMI 1640 medium containing 5% fetal calf serum (FCS) were seeded at 3 x 10^5 cells/well in 96-well flat-bottomed microtiter plates (Becton Dickinson, Lincoln Park NJ, USA) and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial 1:2 dilutions of samples were made in the just-described medium containing 1 $mg \cdot mL^{-1}$ actinomycin D (Banyu Pharmaceutical Co., Tokyo, Japan), and 0.1 mL of each dilution was added to different wells. The following day, the cell survival rate was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol), and 1% sodium dodecyl sulfate was added to each well to solubilize the stained cells. The absorbance of each well was determined at 490 nm, using a microplate reader (Bio-Rad Laboratories, Richmond CA, USA). TNF activity was expressed in units per millilitre, which is the reciprocal of the dilution necessary for 50% lysis of the cells.

The plasma IL-6 concentration was measured in duplicate using a commercially available enzyme-linked immunoassay (IL-6 Enzyme Immunoassay Kit, Advanced Magnetics, Inc., Cambridge MA, USA). The intra- and inter-assay precision was 9% and 6%, respectively, at an IL-6 concentration of 88 $pg \cdot mL^{-1}$. The plasma IL-8 concentration was measured in duplicate using an enzyme-linked immunoassay (IL-8 Enzyme Immunoassay Kit, Advanced Magnetics, Inc., Cambridge MA, USA). The intra- and inter-assay precision was 7% and 4% at an IL-8 concentration of 76 $pg \cdot mL^{-1}$. According to the manufacturer, cross-reactivity with other cytokines is negligible in both assays.

To assess the effect of ketamine on leukocyte viability, different doses (0–1000 µM) of S(+)- or R(-)-ketamine were added to diluted human whole blood and incubated for five hours at 37°C in a 95% air / 5% CO_2 incubator. After incubation, the blood was centrifuged at 700 *G* for ten minutes. Buffy coats were isolated and NH_4Cl lysis of red blood cells was performed. The white blood cells were resuspended in RPMI 1640 medium containing 5% FCS and the cells were stained with 0.2% trypan blue. The cell survival rate was assessed by microscope.

All data are presented as the mean ± SEM. The paired t test was used for statistical analysis to compare values with the control value. For comparison between

two groups, one-way repeated-measures analysis of variance was applied. A significant difference was presumed at a probability value of less than 0.05.

Results

Human whole blood was stimulated using different doses of SEB (0–100 ng·mL^{-1}). SEB induced TNF- production in a dose-dependent manner at concentrations between 0.1 and 10 ng·mL^{-1} in the whole blood. TNF- production reached a plateau with SEB doses of 10 ng·mL^{-1} or more (Figure 1). Therefore, we used an SEB concentration of 10 ng·mL^{-1} in our experiments.

After adding different doses (0–1000 μM) of S(+)- or R(-)-ketamine, whole blood was stimulated with SEB (10 ng·mL^{-1}) (n=10). Figure 2 shows the effects of S(+)- and R(-)-ketamine on SEB-induced TNF- production. When the blood was incubated for six hours, 50 μM S(+)- and R(-)-ketamine significantly suppressed SEB-induced TNF- production (from 373.3 ± 89.2 to 133.3 ± 39.6 U·mL^{-1} for S(+)-ketamine and from 426.7 ± 98.3 to 123.3 ± 43.3 U·mL^{-1} for R(-)-ketamine). S(+)- and R(-)-ketamine significantly suppressed SEB-induced TNF- production in a dose-dependent manner at concentrations between 50 and 1000 μM in comparison with the control (P <0.05). 10 μM S(+)- or R(-)-ketamine had no effect on SEB-induced TNF- production. There were no significant differences between S(+)- and R(-)-ketamine in suppressing SEB-induced TNF- production.

Figure 3 shows the effect of ketamine on SEB-induced IL-6 production. When the blood was incubated for six hours, 100 μM S(+)- and R(-)-ketamine significantly suppressed SEB-induced IL-6 production (from 825 ± 113.1 to 579.7 ± 85.2 pg·mL^{-1} for S(+)-ketamine and from 991 ± 109.1 to 596.3 ± 94.4 pg·mL^{-1} for R(-)-ketamine). At concentrations between 100 and 500 μM, SEB-induced IL-6 production was suppressed by S(+)- and R(-)-ketamine in a dose-dependent manner in comparison with the control (P <0.05). 10 and 50 μM S(+) or R(-)- ketamine had no suppressive effect on SEB-induced IL-6 production. There were no significant differences between S(+)- and R(-)-ketamine in suppressing SEB-induced IL-6 production.

The effect of ketamine on SEB-induced IL-8 production is shown in Figure 4. When the blood was incubated for six hours, 500 μM S(+)- and R(-)-ketamine significantly suppressed SEB-induced IL-8 production (from 1049.2 ± 47.1 to 886.6 ± 31 pg·mL^{-1} for S(+)-ketamine and from 1033 ± 37.6 to 733.7 ± 43.2 pg·mL^{-1} for R(-)-ketamine). As with TNF- and IL-6 production, S(+)- and R(-)-ketamine (500–1000 μM) also suppressed SEB-induced IL-8 production in a dose-dependent manner in comparison with the control (P <0.05). There were no significant differences between S(+)- and R(-)-ketamine in suppressing SEB-induced IL-8 production.

S(+)- and R(-)-ketamine had no effect on L929 cell viability. S(+)- and R(-)-ketamine also had no effect on white blood cell viability, as assessed by the exclusion of the vital stain trypan blue (data not shown).

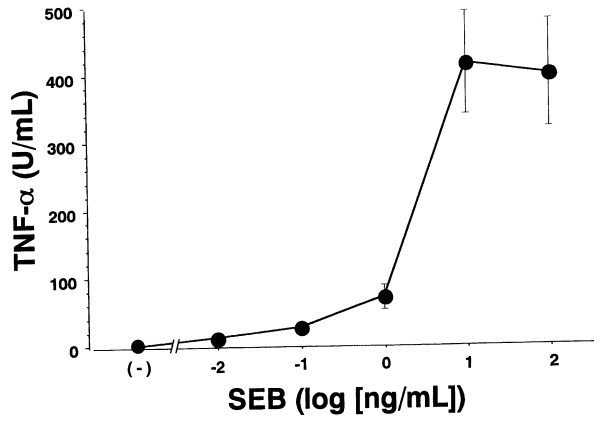

FIGURE 1 Staphylococcal enterotoxin B (SEB)-induced tumour necrosis factor (TNF)- production in human whole blood. Values are expressed as mean ± SEM (n=10).

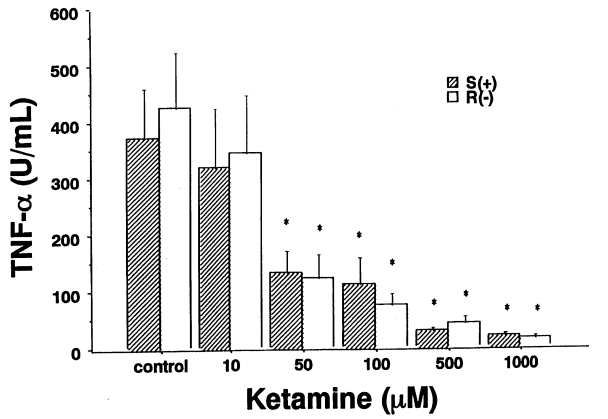

FIGURE 2 Effects of ketamine isomers on staphylococcal enterotoxin B (SEB)-induced tumour necrosis factor (TNF)- production. After ketamine (0–1000 μM) was added, human whole blood was stimulated by SEB (10 ng·mL^{-1}) and incubated for six hours. Values are expressed as mean ± SEM (n=10). *P <0.05 compared with control.

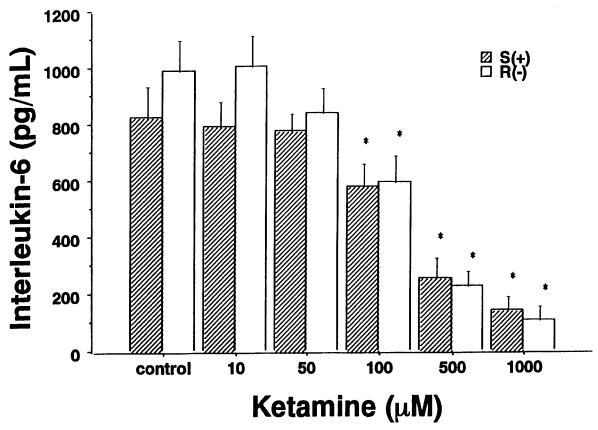

FIGURE 3 Effects of ketamine isomers on staphylococcal enterotoxin B (SEB)-induced interleukin (IL)-6 production. After ketamine (0–1000 μM) was added, human whole blood was stimulated by SEB (10 ng·mL^{-1}) and incubated for six hours. Values are expressed as mean ± SEM (n=10). *P <0.05 compared with control.

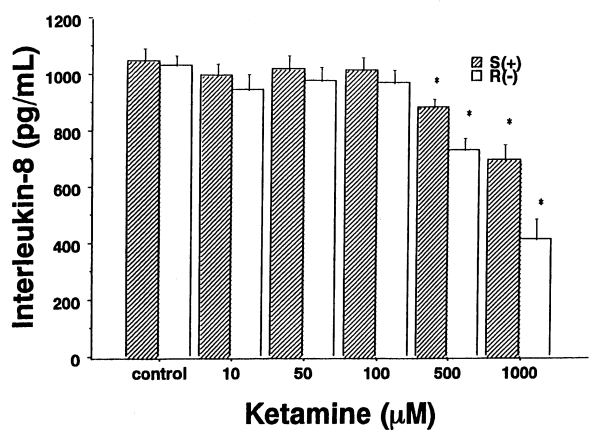

FIGURE 4 Effects of ketamine isomers on staphylococcal enterotoxin B (SEB)-induced interleukin (IL)-8 production. After ketamine (0–1000 μM) was added, human whole blood was stimulated by SEB (10 ng·mL^{-1}) and incubated for six hours. Values are expressed as mean ± SEM (n=10). *P <0.05 compared with control.

Discussion

This study demonstrates that both S(+)- and R(-)-ketamine suppress SEB-induced TNF-, IL-6, and IL-8 production in human whole blood *in vitro*.

Since we used human whole blood as an *ex vivo* model of cytokine production, we could not assess the ability of each cell such as monocytes, macrophages, or T cells on cytokine production. However, this model reduces the confounding factors that may be associated with the isolation of monocytes or neutrophils. Moreover, whole blood is a more physiologic environment.

The production of TNF-, IL-6, and IL-8 increased significantly in human whole blood when stimulated by SEB (Figures 2–4). It has been reported that superantigens stimulate TNF-, IL-6, and IL-8 production in monocytes, macrophages, and T cells.[15–18] As shown in Figures 2, 3, and 4, ketamine suppresses SEB-induced proinflammatory cytokine production in human whole blood. Recently, Yan *et al.*[19] demonstrated that the cells producing SEB- induced TNF- in human whole blood are mainly T cells, and not monocytes or macrophages, suggesting that ketamine may suppress the proinflammatory cytokine production of T cells.

We demonstrated that 50 μM and more S(+)- and R(-)-ketamine suppressed SEB-induced TNF- production and that 100 μM and more S(+)- and R(-)-ketamine suppressed SEB-induced IL-6 production in human whole blood (Figures 2 and 3). Furthermore, 500 μM and more S(+)- and R(-)-ketamine suppressed SEB-induced IL-8 production (Figure 4). The concentration of ketamine in human plasma reaches 110 μM with *iv* administration of ketamine 2.0–2.2 mg·kg^{-1}.[20] This suggests that ketamine might suppress SEB-induced TNF- and IL-6 production at clinical doses.

Ketamine is a racemic mixture (1:1) of two optically active isomers. Ketamine isomers inhibit N-methyl-D-aspartate receptor channels in a stereoselective manner, and this causes their different psychic and analgesic effects.[21] S(+)-ketamine is approximately four times as potent as R(-)-ketamine in its psychomimetic and analgesic effects.[22] Szekely *et al.*[23] demonstrated that neutrophil adherence to the coronary vasculature after ischemia was inhibited by S(+)-ketamine, however, R(-)-ketamine had no effect on it. In our study, there were no differences between the suppressive effects of S(+)- and R(-)-ketamine. Recently, Weigand *et al.*[24] reported that there was no significant difference in the extent of inhibition of N-formyl-methionyl-leucyl-phenylalanine-stimulated CD-18 up-regulation between S(+)- and R(-)-ketamine. These results suggested that this effect of ketamine isomers was not mediated through specific receptor interactions.

In a previous study, we reported that the addition of ketamine two hours after stimulation with LPS effectively suppressed TNF production.[10] We assume that ketamine may regulate LPS-induced TNF production

at a posttranscriptional level. However, the mechanism of the suppressive effect of ketamine on SEB-induced cytokine production in human whole blood still remains unclear. Further studies are needed to elucidate the mechanism of the suppressive effect of ketamine.

In conclusion, we demonstrated that S(+)- and R(-)-ketamine inhibit the SEB-induced production of proinflammatory cytokines, such as TNF-, IL-6, and IL-8, in human whole blood. The suppressive effect of S(+)- ketamine equaled that of R(-)-ketamine.

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