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Ketamine Inhibits Transcription Factors Activator Protein 1 and Nuclear Factor-κB, Interleukin-8 Production, as well as CD11b and CD16 Expression: Studies in Human Leukocytes and Leukocytic Cell Lines

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BACKGROUND: Recent data indicate that ketamine exerts antiinflammatory actions. However, little is known about the signaling mechanisms involved in ketamine-induced immune modulation. In this study, we investigated the effects of ketamine on lipopolysaccharide-induced activation of transcription factors activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) in human leukocyte-like cell lines and in human blood neutrophils.

METHODS: Electric mobility shift assays were used to investigate ketamine's effects on nuclear binding activity of both transcription factors in U937 cells, and a whole blood flow cytometric technique was used for AP-1 and NF-κB determination in leukocytes. Cell lines with different expression patterns of opioid and *N*-methyl-D-aspartate receptors were used for reverse transcription–polymerase chain reaction to investigate receptors involved in ketamine signaling. Ketamine's effect on interleukin-8 production was assessed in a whole blood assay.

RESULTS: Ketamine inhibited both transcription factors in a concentration-dependent manner. These effects did not depend on opiate or *N*-methyl-D-aspartate receptors. Ketamine also reduced interleukin-8 production in whole blood and expression of CD11b and CD16 on neutrophils.

CONCLUSION: The immunoinhibitory effects of ketamine are at least in part caused by inhibition of transcription factors NF-κB and AP-1, which regulate production of proinflammatory mediators. However, signaling mechanisms different from those present in the central nervous system are responsible for ketamine-mediated immunomodulation. (Anesth Analg 2010;110:934–41)

The phencyclidine ketamine is widely used in anesthesia and intensive care. In addition to its effects on the central nervous system (CNS), ketamine has been found to act as an immunomodulator. In particular, ketamine inhibits leukocyte expression of adhesion molecules,[1] phagocytosis,[2] and proinflammatory cytokine production.[3] It has also been demonstrated that ketamine reduces nuclear factor-κB (NF-κB) activation in endotoxemic shock and thereby attenuates lipopolysaccharide (LPS)-induced damage to liver, stomach, and lungs.[4–6] Two studies indicate that calcium and adenosine are involved in the regulation of NF-κB activity by ketamine.[7,8] However, it remains unclear by which signaling mechanism ketamine reduces the NF-κB response in different leukocyte populations.

Neutrophils are key cells in the innate immune response observed during inflammatory processes. After activation, these cells display an upregulated expression of adhesion molecules[9] and surface receptors. CD11b and CD16 facilitate phagocytosis of particles and microorganisms, which have been opsonized with complement fragments or immunoglobulins. In addition to their enhanced phagocytic activity, the production of proinflammatory cytokines is increased in neutrophils after stimulation.[10] Neutrophils also play a major role in organ dysfunction observed during endotoxemic shock. In this regard, modulation of neutrophil activity by ketamine could contribute to the reduction in tissue damage observed after ketamine administration.[11]

The production of proinflammatory cytokines such as interleukin (IL)-8, IL-6, and tumor necrosis factor (TNF)-α, depends on activation of the transcription factor NF-κB.[12] In the cytoplasm, NF-κB homo- and heterodimers, consisting of p65 and the p50 subunits, are bound to the NF-κB inhibitor I κ B-α (IκBα). In response to LPS, IκBα is rapidly degraded and enables nuclear translocation of the p65/p50 heterodimer, which initiates transcription of the above-mentioned genes.[13]

Activator protein 1 (AP-1) represents another transcription factor that is involved in transcriptional regulation of inflammatory proteins such as IL-8 and monocyte chemoattractant protein-1.[14,15] The AP-1 complex is composed of

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either homo- or heterodimers, which consist of protein products derived from the proto-oncogenes c-*fos* and c-*jun*.[16] Modulation of transcriptional activity by ketamine is likely to be involved in ketamine-induced attenuation of organ damage during endotoxemia.[11]

Several antiinflammatory properties of ketamine, mainly a reduction of cytokine production, are possibly related to transcriptional changes. However, other alterations in immune function, such as a decrease in phagocytic activity and oxidative burst,[2,17,18] might rather be caused by other mechanisms, including changes in the expression of surface receptors, which facilitate phagocytosis of microorganisms, such as CD16 and CD11b.

Therefore, in this study, we aimed to identify signaling pathways involved in ketamine-induced modulation of neutrophil activity. We hypothesized that ketamine (1) influences the transcriptional activity of NK-κB and AP-1 and (2) reduces the expression of leukocyte integrins and phagocytosis-related surface molecules.

METHODS

Cell Culture, Reagents, and Induction

The monocytic cell line U937 and the promyelocytic cell line HL-60 were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, HEPES (2.38 g/L), $NaHCO_3^-$ (2 g/L), L-glutamine (0.3 g/L), pyruvic acid (0.088 g/L), glucose (4.5 g/L), penicillin (100 U/mL), and streptomycin (100 g/L) at 37°C in a humidified 5% CO_2/95% air atmosphere. The monocytic cell line U937 was used to study transcriptional activity. HL-60 cells were used to obtain μ-opioid receptor–positive cells. To differentiate HL-60 cells into the granulocytic phenotype, cells were treated for 5 days with 1 μM retinoic acid, with differentiation being confirmed by nitroblue tetrazolium reducing ability and response to *N*-formyl-methionyl-leucyl-phenylalanine. A total of 10^7 cells were incubated for 2.5 hours with ketamine hydrochloride (Sigma Chemical Co., St. Louis, MO) as indicated, followed by a 30-minute induction of transcription factors by LPS 100 ng/mL (*Escherichia coli* serotype O26:B6, Sigma Chemical Co.). We chose 2 concentrations of ketamine, 100 μM and 1 mM. Plasma concentrations of >100 μM were reported after ketamine injection in patients[19] and were therefore regarded as clinically relevant, and 1 mM was chosen as a supraclinical concentration.

Preparation of Cytosolic and Nuclear Extracts

Cytosolic and nuclear extracts of U937 and HL-60 cell pellets were obtained by a modification of the method described by Schreiber et al.[20] Briefly, cell pellets were washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in 150 μL buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethanesulphonylfluoride). After a 10-minute incubation on ice, cell lysis was checked microscopically. After centrifugation (16,000*g*, 30 seconds, 4°C), the supernatant containing the cytoplasmic protein fraction was collected. The remaining pellet was washed in PBS, resuspended in 50 μL buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethanesulphonylfluoride), and incubated on ice for 20 minutes. After centrifugation (16,000*g*, 2 minutes, 4°C), the supernatant containing the nuclear protein fraction was collected. Protein concentrations of nuclear extracts were determined by the BioRad Protein Assay (BioRad Laboratories, Hercules, CA). The samples were aliquoted and stored at −70°C until further analysis. Aliquots of the same nuclear extracts were used for NF-κB and AP-1 determination, respectively.

Electric Mobility Shift Assay

Nuclear protein 5 μg was preincubated for 20 minutes at 22°C with radiolabeled oligonucleotide, poly[dI-dC], and poly-L-lysine in reaction-buffer (100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)2SO_4$, 5 mM DTT, 1% Tween 20, and 150 mM KCl). The double-stranded oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) contained the DNA binding site for NF-κB (underlined): 5′ AGT TGA GGG GAC TTT CCC AGG C 3′ and AP-1, respectively: 5′ CGC TTG ATG ACT CAG CCG GAA 3′. After incubation, DNA-protein complexes were resolved by electrophoresis on 7.5% polyacrylamide gels under nondenaturing conditions. Gels were dried for 1 hour at 80°C before autoradiography with intensifying screens.

Isolation of Total RNA and Reverse Transcription–Polymerase Chain Reaction

After treatment with ketamine and subsequent stimulation with LPS for 2 hours, cells were collected, centrifuged, and homogenized in 1 mL TRI Reagent (Molecular Research Center, Cincinnati, OH) for 5 minutes. After addition of 0.1 mL 1-bromo-3-chloropropane, samples were vigorously mixed and centrifuged for 15 minutes at 12,000*g*. The aqueous phase was transferred into a fresh tube. RNA was precipitated with isopropanol, washed with 75% ethanol, and air-dried for 10 minutes. After resuspension in RNase-free water, RNA was analyzed spectrophotometrically and electrophoresed on a 1% agarose gel to ensure purity.

After denaturation at 95°C, 1 μg neutrophil RNA and 3 μg RNA from both cell lines were reverse transcribed at 42°C using SuperScript II RNase H–reverse transcriptase and random hexamer primers (Both Gibco BRL, Gaithersburg, MD). Ten microliters of the reverse-transcription product was added to the polymerase chain reaction (PCR) mix containing specific primers for either the μ-opiate receptor or *N*-methyl-D-aspartate (NMDA)-1 receptor. Taq DNA polymerase (Gibco BRL, Gaithersburg, MD) was used to catalyze the reaction. All PCR reactions were followed by an extension cycle at 72°C for 10 minutes. The following primers were used: NMDA-1 sense: 5′ GAT GTC TTC CAA GTA TGC GGA 3′, antisense: 5′ GGG AAT CTC CTT CTT GAC CAG 3′[21]; μ receptor sense: 5′ GGT ACT GGG AAA ACC TGC TGA AGA TCT GTG 3′, antisense: 5′ GGT CTC TAG TGT TCT GAC GAA TTC GAG TGG 3′.[22] PCR conditions were as follows: NMDA-1: 30 cycles (95°C, 30 seconds; 50°C, 30 seconds; and 72°C, 30 seconds), amplifying a 667–base pair (bp) fragment, μ receptor: 30 cycles (95°C, 1 minute; 53°C, 1 minute; and 72°C, 1 minute), amplifying a 441-bp fragment. Total RNA from SH-SY5Y neuroblastoma cells known to express NMDA and μ-opiate receptors served as a positive control.

Whole Blood Experiments

For measurement of surface receptor expression, flow cytometric quantification of transcription factors and determination of IL-8 whole blood was used. Venous blood samples were obtained from healthy male volunteers aged 20 to 40 years. Written consent was obtained from all participants, and the study was approved by the local ethics committee. Blood samples were incubated with racemic ketamine (Parke-Davis, Berlin, Germany) for different time intervals (15 and 180 minutes) and stimulated with LPS (10 ng/mL) for 30 minutes. For each series of experiments, a fresh dilution of ketamine was prepared, and no additives apart from sodium chloride and 1 N hydrochloric acid were contained in the commercially available ketamine preparation.

Flow Cytometric Determination of Surface Receptor Expression

After incubation with the above-mentioned agents, whole blood samples were stained with mouse-derived monoclonal antibodies. Single-color reagents were fluorescein isothiocyanate (FITC)-conjugated CD11b (Clone ICRF 44), CD35 (Clone E 11), and CD16 (Clone LNK 16, Camon/Serotec, Wiesbaden, Germany). Nonfluorescent antibodies against CD11b, CD16, and CD35 were used to reach optimal setups at the flow cytometer (FacsCalibur, Becton Dickinson, Heidelberg, Germany). Cell surface staining was performed by incubating 100 mL blood with 10 mL of the appropriate antibody reagent for 15 minutes in the dark at room temperature. Lysing solution (Facs®, Brand Lysing Solution, Becton Dickinson) was used for red cell lysis. Samples were washed twice with PBS and stored on ice in the dark until flow cytometric analysis. After staining of cell nuclei with propidium iodide, flow cytometric analysis was performed within 30 minutes.

Flow Cytometric Quantification of Transcription Factors

For further validation and comparison of a flow cytometric technique with the electric mobility shift assay (EMSA) as the usual method for evaluation of transcription factors, NF-κB and AP-1 nuclear binding were also determined by flow cytometry in ketamine-treated U937 cells with or without LPS stimulation. Flow cytometric detection of transcription factors was performed as described elsewhere in great detail.[23,24]

Briefly, U937 cells were incubated with racemic ketamine for 2.5 hours at different concentrations. Controls were incubated with 0.9% saline. To induce transcription factors AP-1 and NF-κB, samples were stimulated with 100 ng/mL LPS for 30 minutes in a 37°C water bath. Controls were set up with PBS. The cells were centrifuged at 200*g* for 5 minutes and washed once with PBS before staining of nuclei using a commercially available DNA staining kit (Cycletest Plus DNA Reagent Kit, Becton Dickinson). After centrifugation, pellets were washed twice in 3 mL citrate buffer. Cells were resuspended in a mixture of 250 μL solution A (trypsin in a spermine tetrahydrochloride detergent buffer) and 200 μL solution B (trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride) and incubated for 10 minutes at room temperature. A total of 40 μL of anti–NF-κB p65 or AP-1 polyclonal rabbit antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was added, and cells were incubated for 10 minutes at 22°C. A total of 2.5 μL of a FITC-conjugated anti-rabbit monoclonal antibody (Sigma Chemical Co., Deisenhofen, Germany) was added and incubated for 10 minutes at room temperature. Propidium iodide solution 200 μL was added to the aliquots and incubated for 10 minutes.

Stained nuclei were detected by the fluorescence 2 (FL2) filter. The FL1 detector was used to detect NF-κB activation by measuring emission of green fluorescence at 530 nm corresponding with FITC staining. Fluorescence due to unspecific binding of the secondary antibody was excluded by incubation of whole blood with FITC-labeled immunoglobulin G alone. Commercially available DNA particles (DNA Quality Control Particles Kit, Becton Dickinson) were used for the flow cytometer setup. Twenty thousand events were recorded for each sample. Cells were analyzed for FITC staining using histogram analysis of the FL1 parameter. The median of channel fluorescence was used as an indicator for the intensity of nuclei fluorescence.

Enzyme-Linked Immunosorbent Assay

Whole blood was aliquoted into tissue culture plates, diluted 1:5 with RPMI, and incubated with ketamine and LPS as indicated. Samples were centrifuged, and the supernatant was collected. IL-8 concentrations were determined using a commercially available kit (OptEIA-Set human IL-8, Pharmingen, San Diego, CA).

Statistical Analysis

Statistical analysis was performed using R for Windows version 2.8.0. Friedman test followed by Wilcoxon- Wilcox procedure, allowing multiple comparisons between groups, was used to analyze changes in IL-8 concentration and receptor expression.

RESULTS

To explore possible mechanisms of ketamine on transcriptional activity, we started with experiments on leukocytic cell lines. We incubated these cells for 2.5 hours with ketamine followed by a 30-minute stimulation with LPS to increase NF-κB and AP-1 transcriptional activity.

Ketamine Inhibits LPS-Induced NF-κB and AP-1 Activity in U937 Cells

As shown by EMSA, ketamine reduced NF-κB binding in U937 cells to a consensus oligonucleotide at both concentrations tested (Fig. 1). Similar results were observed in HL-60 cells (data not shown). Although this inhibition was present after treatment with 100 μM ketamine, it was more pronounced at the higher concentration of 1 mM (Fig. 1A). Similar results were obtained by a flow cytometric determination of nuclear content of NF-κB (Fig. 1B).

Similar to the results obtained for NF-κB, EMSA for AP-1 revealed that nuclear binding was also reduced after incubation with 100 μM or 1 mM ketamine and subsequent LPS stimulation (Fig. 2). The maximal inhibition was observed after 30 minutes of stimulation with LPS. We detected a small amount of AP-1 binding activity even in our control sample. Interestingly, a suppression of this

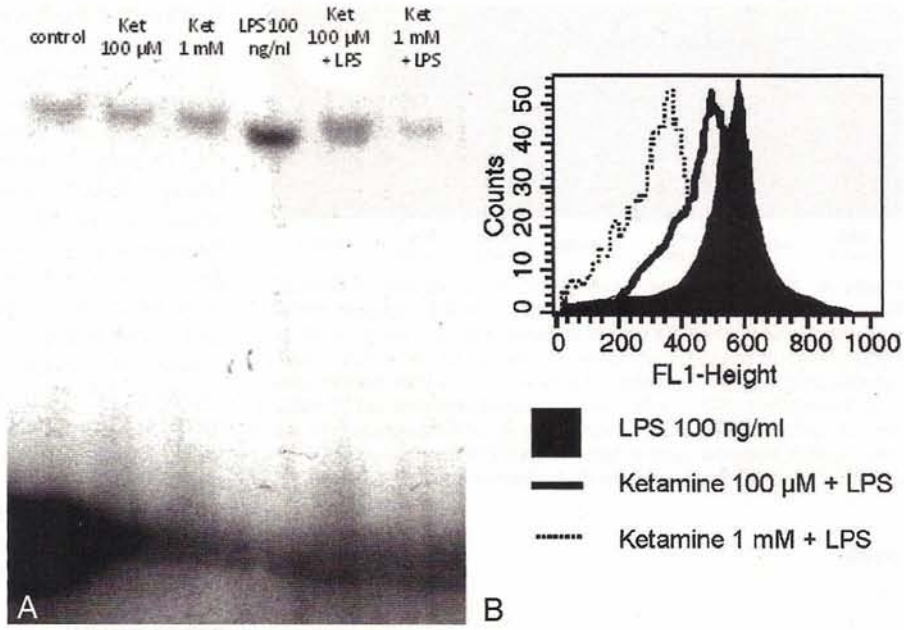

Figure 1. A, Ketamine (Ket) decreases nuclear factor-κB (NF-κB) DNA binding activity as determined by electric mobility shift assay (EMSA). U937 cells were incubated with 100 µM or 1 mM ketamine for 2.5 h, respectively, followed by stimulation with 100 ng/mL lipopolysaccharide (LPS) for 30 min. Ketamine reduced NF-κB DNA binding activity in a concentration-dependent manner. Results were comparable in 3 independent experiments. B, Flow cytometric determination of NF-κB content shows a decrease after treatment with 1 mM ketamine for 10 min and subsequent stimulation with LPS.

Figure 2. Ketamine (Ket) decreases activator protein 1 (AP-1) binding activity as determined by electric mobility shift assay. U937 cells were incubated with 100 M or 1 mM ketamine for 2.5 h, followed by stimulation with 100 ng/mL lipopolysaccharide (LPS) for 30 min. Ketamine reduced AP-1 DNA binding activity in a concentration-dependent manner. Results were comparable in 3 independent experiments.

AP-1 binding activity was observed after ketamine treatment with both concentrations in unstimulated samples.

Similar results demonstrating a decrease in NF-κB and AP-1 activity were obtained for opioid receptor–positive HL-60 cells differentiated into a granulocytic phenotype (data not shown).

Ketamine's Inhibitory Effects Are Not Mediated by µ-Opiate or NMDA-1 Receptors

In an attempt to further elucidate the signaling processes involved in ketamine's immunoinhibiting actions, we used reverse transcription–PCR to screen for the expression of µ-opioid and NMDA-1 receptors. Both receptors are constitutively expressed in SH-SY5Y neuroblastoma cells.[25,26] After treatment with retinoic acid, HL-60 cells produced µ-opiate receptor mRNA, whereas only a very faint band was found in undifferentiated HL-60 cells (Fig. 3). This weak expression of µ-opioid receptor RNA may be attributed to spontaneous differentiation occurring in approximately 10% of HL-60 cells.

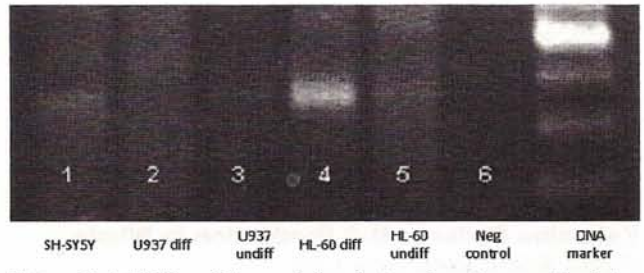

Figure 3. Inhibition of transcription factors is not caused by interaction of ketamine with µ-opioid receptors on immune cells. Despite inhibition of transcription factors by ketamine, U937 cells did not express µ-opioid receptor mRNA. In contrast, HL-60 cells expressed a 441–base pair (bp) band correlating with µ-opioid receptor mRNA. Lane 1: SH-SY5Y neuroblastoma cells (positive control), lane 2: differentiated U937 cells, lane 3: undifferentiated U937 cells, lane 4: differentiated HL-60 cells, lane 5: undifferentiated HL-60 cells, lane 6: negative control, last lane: 100-bp DNA marker. One representative experiment of 3 is shown.

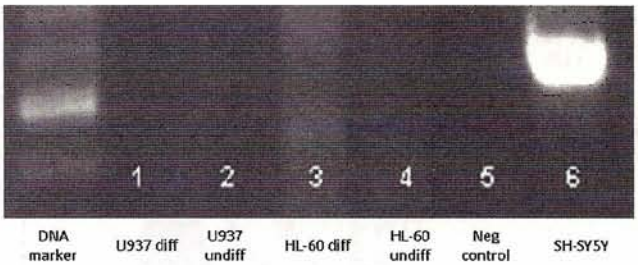

Figure 4. *N*-methyl-D-aspartate (NMDA)-1 receptors are not involved in transcriptional inhibition by ketamine. NMDA-1 receptor mRNA was only found in SH-SY5Y neuroblastoma cells, but not in HL-60 or U937 cells, indicating that ketamine exerts its inhibitory action independent from this receptor. First lane: 100-bp DNA marker, lane 1: differentiated U937 cells, lane 2: undifferentiated U937 cells, lane 3: differentiated HL-60 cells, lane 4: undifferentiated HL-60 cells, lane 5: negative control, lane 6: SH-SY5Y neuroblastoma cells (positive control). One representative experiment of 3 is shown.

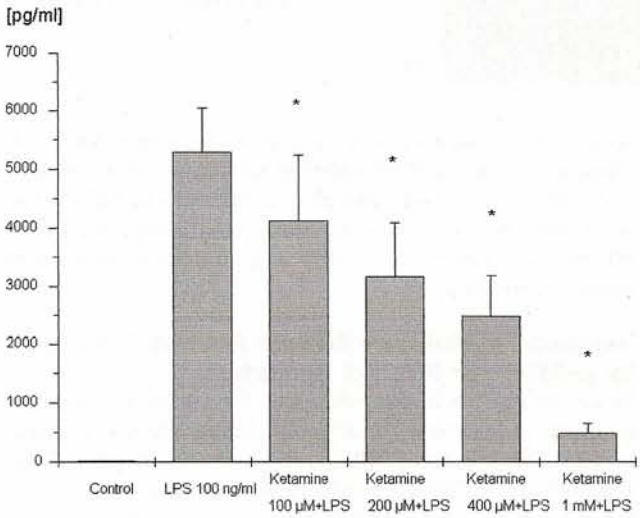

Figure 5. Interleukin-8 concentrations after incubation of whole blood with different concentrations of ketamine for 15 min and subsequent stimulation with lipopolysaccharide (LPS) for 30 min. Mean and SD of 6 experiments are shown. *Significant differences compared with LPS stimulation ($P < 0.05$).

In contrast, μ-opioid receptor mRNA was not detected in U937 cells after treatment with retinoic acid. Neither LPS nor retinoic acid could induce NMDA-1 receptor mRNA in either cell line (Fig. 4). Because ketamine attenuated AP-1 and NF-κB in both cell types, these effects were mediated independently from μ-opiate and NMDA-1 receptors, suggesting a novel receptor-mediated process or a signal transduction mechanism independent from surface receptors.

Ketamine Reduces IL-8 Production in Whole Blood Stimulated with LPS

The mean value of IL-8 determined in the plasma of unstimulated human whole blood from healthy volunteers was 9.2 ± 3 pg/mL. After stimulation with LPS, the IL-8 concentration increased to 5285 ± 748 pg/mL. Ketamine suppressed the LPS-induced IL-8 production in a dose-dependent manner at concentrations between 100 μmol/L and 1 mmol/L (Fig. 5). Concentrations <100 μmol/L exhibited no effect (data not shown).

Ketamine Reduces Expression of Neutrophil Surface Receptors CD16 and CD11b

To further characterize the modulation of the leukocyte activation by ketamine in a more clinically relevant model, we studied the effect of racemic ketamine on neutrophil CD11b and CD16 expression after stimulation of whole blood with LPS (10 ng/mL). We used 2 different incubation times for ketamine (15 and 180 minutes) to discriminate between short-term and longer-term effects. To investigate concentration-dependent effects, we incubated whole blood with 100 μM ketamine as a clinically relevant concentration[19] and 1 mM ketamine as a supraclinical concentration. In the absence of ketamine, LPS induced a significant increase in expression of both receptors (Fig. 6, A and B). Ketamine incubation for either 15 or 180 minutes significantly reduced the LPS-stimulated increase in CD11b receptor expression (Fig. 6A); however, LPS-stimulated CD16 expression was only inhibited by a supraclinical concentration of 1 mM ketamine (Fig. 6B). No effect on surface receptor expression was noted in concentrations of <100 μM (data not shown).

DISCUSSION

Our data indicate that ketamine reduces LPS-stimulated surface receptor expression, attenuates LPS-induced activation of transcription factors NF-κB and AP-1, and may thereby decrease the production of proinflammatory mediators. Both transcription factors are involved in regulation of cell growth and differentiation as well as in regulation of inflammatory responses. Transcriptional activation and repression of transcription factors by anesthetic drugs have been thoroughly investigated in leukocytes. It has become clear that ketamine exerts its antiinflammatory actions primarily via inhibition of leukocyte reactivity without modulating endothelial responses.[27] In accordance with these results, a reduction of NF-κB activity by ketamine has been demonstrated in the intestine as well as in monocytes.[7,28] Furthermore, a decrease in production of NF-κB- and AP-1-dependent mediators IL-8, IL-6, and TNF-α has been reported after pretreatment of whole blood with ketamine and subsequent stimulation with LPS.[3] However, the role of ketamine in inhibiting neutrophil function has not been investigated thoroughly, and the intracellular mechanisms involved remain unclear. In this study, we demonstrated the interaction of ketamine with transcription factors NF-κB and AP-1 not only in a monocytic cell line but also in human neutrophils. In addition, we showed that ketamine inhibits the production of proinflammatory mediators and the expression of surface receptors as a key mechanism, which may contribute to the immunoinhibition induced by ketamine.

Regulation of the NF-κB activity in immunocytes is tightly controlled by IκBα. LPS induces rapid phosphorylation of IκBα.[29] After degradation of IκBα, NF-κB subunits p50/p65 are released and translocated as transcriptionally active heterodimers into the nucleus. Inhibitors of NF-κB can target different steps within the activation process of NF-κB. Although IL-10 and IL-13[30] as well as glucocorticoids[31] diminish NF-κB activity by induction of IκBα mRNA, salicylates block the LPS-induced proteolytic degradation of IκBα thereby preventing the nuclear translocation of c-Rel/p65 heterodimers.[32] Further studies must

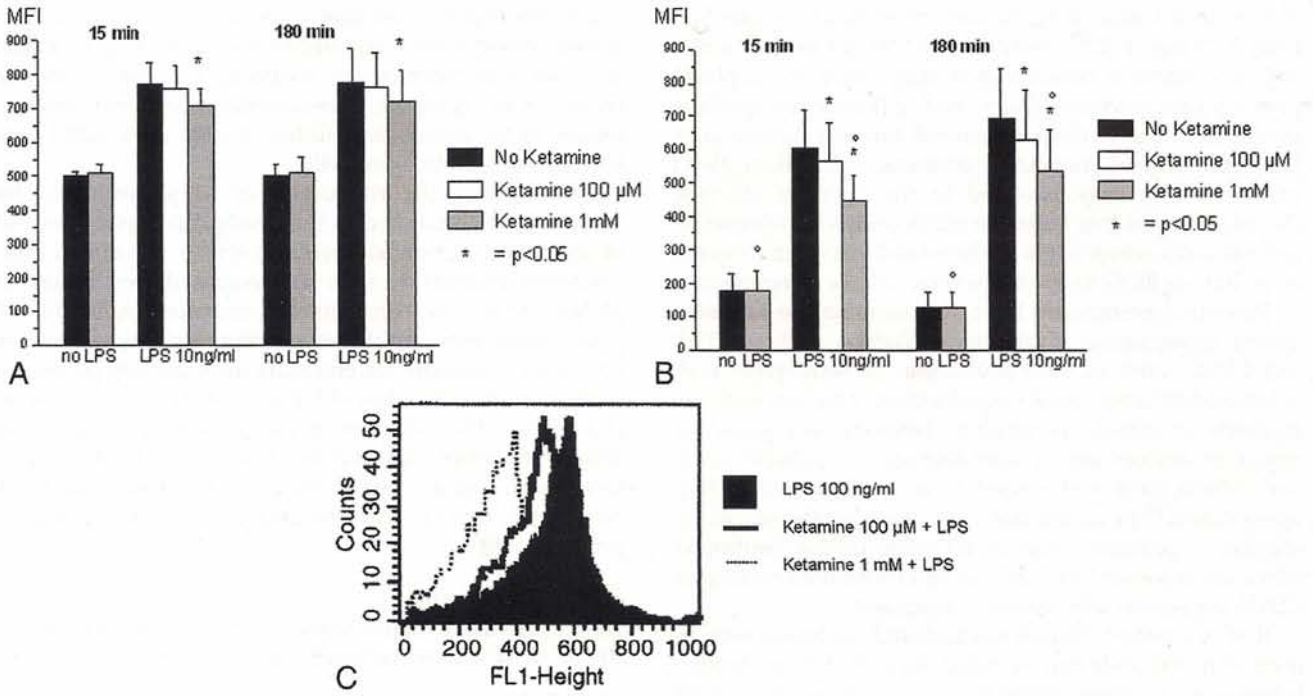

Figure 6. Flow cytometric determination of ketamine effects on surface receptor expression on human neutrophils in a whole blood assay. A, Expression of surface receptor CD16 with and without lipopolysaccharide (LPS) stimulation after 15 and 180 min incubation with clinically relevant and supraclinical concentrations of ketamine. B, Expression of surface receptor CD11b with and without LPS-stimulation after 15 and 180 min incubation with clinically relevant and supraclinical concentrations of ketamine. C, Original flow cytometric determination of neutrophils treated with ketamine for 15 min and/or LPS as indicated.

elucidate whether changes in IκBα concentration or interaction of ketamine during IκBα degradation might play a role in ketamine-induced inhibition of NF-κB.

Interestingly, we observed a reduction of AP-1 binding activity in unstimulated U937 cells treated with ketamine. However, we did not find a reduction in nuclear AP-1 content in neutrophils after treatment with ketamine as determined in whole blood assays. This could indicate that ketamine may display a direct inhibitory effect on DNA binding rather than interfere with the nuclear translocation process of AP-1. Further studies are needed to clarify the clinical relevance of this observation.

The exact cellular mechanisms leading to ketamine-related NF-κB attenuation in immune cells have not been fully investigated. Although in the CNS ketamine's effects are considered to be mediated mainly by antagonism at NMDA receptors[33] or by interaction with opioid receptors,[34] these receptors were not related to the inhibition of transcription factors and proinflammatory mediators in our experiments. The fact that ketamine induced inhibition of AP-1 and NF-κB in a cell line negative for both receptors indicates that the immunomodulating properties of ketamine are either transmitted by signaling processes different from those present in the CNS or by a different class of receptors. In this context, it is noteworthy that ketamine in the brain not only binds to opioid and NMDA receptors but also interacts with σ receptors.[35] σ receptors have been defined as non-opiate, nondopaminergic, and nonphencyclidine receptors and are also present on immunocytes including neutrophils.[36] Expression of the σ1 receptor subtype is particularly abundant in cells of the immune and endocrine systems and agonists such as neuropeptide Y inhibit phagocytosis via direct and centrally mediated effects.[36] Hence, ketamine might mediate its effects on neutrophil function via this class of receptors. Further mechanisms by which ketamine might influence cellular function include the blockade of voltage-operated sodium channels, an effect that has been demonstrated in skeletal muscle and neurons,[37] but not yet in immune cells. Thus, it remains to be elucidated whether the immunoinhibition induced by ketamine depends on surface receptors on immunocytes or constitutes receptor-independent signal transduction mechanism such as ion channels. Furthermore, the clinical relevance of these findings needs to be evaluated in in vivo studies.

We used 2 different ketamine concentrations (100 µM and 1 mM) for our experiments. Concentrations of >25 µg/mL (equivalent to approximately 100 µM) have been reported after IV injection of ketamine at a dose of 2 to 2.2 mg/kg body weight[19] and can therefore be regarded as clinically relevant. In addition, we took into consideration the plasma protein binding of ketamine, which is reported to be 60%.[38] Thus, the dose of ketamine applied in a whole blood assay should presumably be at least between 150 and 200 µM to reach a concentration of unbound ketamine of 100 µM. Furthermore, the concentrations we used allow comparisons with previous studies performed with similar ketamine concentrations.[1,37,39,40] To differentiate between rapidly occurring changes, e.g., for receptor expression, and longer-time effects of ketamine on downstream processes such as cytokine production, we chose incubation times between 15 and 180 minutes. In a previous study, diverging effects of ketamine on macrophage function have

been reported depending on concentration and incubation time.[39] Chang et al.[40] described that 100 μM ketamine as a clinically relevant concentration suppressed macrophage phagocytosis, oxidative ability, and inflammatory cytokine production. The authors concluded that the reduction of the mitochondrial membrane potential rather than direct cellular toxicity may have led to the observed changes. Overall, it seems that ketamine elicits complex responses in immunocytes, which might not be related to a specific mechanism, but are likely to involve several cellular components.

Previous investigations have demonstrated that ketamine inhibits upregulation of adhesion molecules, such as CD18 and CD62L after in vitro stimulation of neutrophils with *N*-formyl-methionyl-leucyl-phenylalanine and phorbol 12-myristate 13-acetate.[1] In addition, ketamine had protective effects on hemorrhage-induced leukocyte-endothelial adhesion, which is in part related to an inhibition of CD11b upregulation.[41] In accordance with these results, our study suggests a protective role of ketamine during neutrophil activation as shown by a reduction of LPS-induced neutrophil CD11b expression after ketamine treatment.

IL-8 is a potent chemoattractant and facilitates recruitment of neutrophils into inflamed tissue.[42] IL-8 production is regulated by transcription factors NF-κB and AP-1, both of which are activated in response to LPS.[15] Different cell types such as T lymphocytes, monocytes, neutrophils, epithelial, and endothelial cells secrete IL-8 upon stimulation. Plasma levels of IL-8 are increased in bacteremia and sepsis.[43] Our results demonstrate inhibition of LPS-induced IL-8 production by ketamine in a whole blood assay, which again suggests an antiinflammatory role of ketamine in systemic inflammation.

Some rat models of sepsis indicate that the antiinflammatory effects of ketamine even in subanesthetic doses may contribute to improved survival.[44–47] The beneficial effects of ketamine are probably achieved through interference with the inflammatory cascade, in particular by inhibition of NF-κB- and AP-1-regulated transcription, as evidenced by our results as well as previous studies demonstrating the attenuation of the proinflammatory markers such as IL-6, IL-8, and TNF-α. In addition, some investigators found that administration of ketamine dose-independently inhibited hypotension, metabolic acidosis, and cytokine responses in rats injected with endotoxin,[48,49] although these results were not confirmed by all investigators.[50,51] Because ketamine was also shown to inhibit the production of proinflammatory cytokines in endotoxin-stimulated human leukocytes,[1,3,39,52] it may also deploy favorable effects in human sepsis.

However, there are some limitations to our study design. First, results obtained in cell culture or ex vivo whole blood experiments may not fully reflect changes observed in patients. This may in particular be the case for the longer incubation time, which as an ex vivo experiment cannot consider the pharmacologic kinetics occurring in the body, including distribution and redistribution of ketamine as well as changes in plasma concentrations as a consequence thereof. Second, the interaction of neutrophils with the endothelium was not investigated. In our experiments, activated neutrophils remained in the whole blood, whereas in vivo endothelial cells facilitate transmigration of neutrophils into the tissue by expression of selectins and adhesion molecules. Third, although we were able to demonstrate the inhibition of transcriptional activity and neutrophil function by ketamine, the molecular mechanisms, including the role of specific receptors, ion channels, and intracellular enzyme activities remain to be investigated further to fully understand how ketamine acts on immune cells.

In summary, the attenuation of NF-κB and AP-1 by ketamine demonstrated in this study can explain several of the major immunosuppressive effects associated with ketamine treatment, such as decreased expression of phagocyte surface receptors and reduced production of proinflammatory cytokines. Further investigations are necessary to completely elucidate the signaling processes leading to the inhibition of NF-κB and AP-1 by ketamine. In addition, the influence of this anesthetic drug on the activity of other transcription factors involved in cytokine regulation and acute immune response remains to be investigated in in vitro and in vivo inflammatory processes. ■

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