Midazolam Inhibits Proinflammatory Mediators in the Lipopolysaccharide-activated Macrophage
Seon Nyo Kim, B.Sc.,* Soo Chang Son, M.D., Ph.D.,† Sang Mook Lee, M.D.,‡ Cuk Seong Kim, Ph.D.,§ Dae Goon Yoo, B.Sc.,* Sang Ki Lee, Ph.D.,§ Gang Min Hur, M.D., Ph.D.¶ Jin Bong Park, D.V.M., Ph.D.,# Byeong Hwa Jeon, M.D., Ph.D.#

**Background:** Midazolam, a benzodiazepine, has a hypnotic effect and is widely used as a sedative. The role of midazolam in activation of macrophages during sepsis is not known. The aim of this study was to evaluate the antiinflammatory actions of midazolam in cultured macrophages.

**Methods:** Using a macrophage cell line, RAW264.7 cells, the effect of midazolam on proinflammatory mediators and activation of mitogen-activated protein kinase was measured by Western blot. Nuclear factor-kB (NF-kB) activation and translocation of p65 subunit of NF-kB was measured using luciferase assay and immunocytochemistry. Superoxide production was measured by lucigenin chemiluminescence.

**Results:** Midazolam significantly inhibited lipopolysaccharide-induced up-regulation of both cyclooxygenase 2 and inducible nitric oxide synthase in a dose-dependent manner (approximately 3–30 µM). IkB-α degradation and NF-kB transcriptional activity induced by lipopolysaccharide were also suppressed by the midazolam. Nuclear translocation of the p65 subunit of NF-kB was inhibited by midazolam. Furthermore, midazolam suppressed phosphorylation of p38 mitogen-activated protein kinase and also inhibited lipopolysaccharide-induced superoxide production in macrophages.

**Conclusions:** These results suggest that midazolam has an antiinflammatory action by inhibiting inducible nitric oxide synthase and cyclooxygenase-2 expression, possibly through suppression of NF-kB and p38 mitogen-activated protein kinase activation.

SEPSIS is one of the most important causes of multiple organ failure and is still associated with a high mortality rate. Lipopolysaccharide, a gram-negative bacterial outer membrane component, has been implicated as a critical factor contributing to the pathogenesis of sepsis. Activated macrophages contribute to inflammatory responses and can produce and release several cytokines into the general circulation to exert systemic effects that occur during sepsis. Proinflammatory mediators such as cytokines and nitric oxide play pivotal roles in various inflammatory diseases. In septic shock, excessive production of nitric oxide has been proposed to be a major factor involved in tissue damage. Cyclooxygenase 2 (COX-2) or inducible nitric oxide synthase (iNOS) is induced in macrophages by proinflammatory stimuli, including mitogens, cytokines, and bacterial lipopolysaccharide. Accumulated data indicate that COX-2 and iNOS are involved in many inflammatory processes and are induced in various carcinomas, suggesting that COX-2 and iNOS play a key role in inflammation and tumorigenesis. Therefore, modulating these inflammatory factors may influence lipopolysaccharide-induced sepsis.

Midazolam, a benzodiazepine derivative, is the most widely used anesthetics for sedation. Although macrophages are the important effector cells for immunity, excessive production of cytokines in the activated macrophage can induce a systemic inflammatory response. Some lines of evidence imply interaction between midazolam and proinflammatory mediators. Peripheral benzodiazepine binding sites exist on the mouse macrophages as well as other tissues. Midazolam interferes with the synthesis and release of nitric oxide and tumor necrosis factor α (TNF-α) generated by activated microglial cells, blood monocytes, and mast cells, suggesting an inhibitory action on proinflammatory mediators. However, Rossano et al. reported that anesthetics such as propofol, midazolam, and pentothal sodium induced the release of cytokines by human monocytes and lymphocytes in vitro. Therefore, the functional role of midazolam on the activation of macrophages has not been fully clarified.

We hypothesized that midazolam serves to suppress macrophage activation via inhibiting of proinflammatory mediators in the macrophage cells. We investigated the effect of midazolam on the proinflammatory mediators, iNOS and COX-2, and its underlying mechanism in the lipopolysaccharide-activated macrophage cells.

**Materials and Methods**

**Cell Culture and Reagent**
Murine macrophage cell line RAW264.7 was used throughout the experiments. The RAW264.7 cell line is an immortalized macrophage clone isolated from BALB/c mice (Nrramp1<sup>+/−</sup>) transformed with Abelson leukemia virus. RAW264.7 cells and the human embryonic kidney 293 cell line were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained at subconfluence in a 95% air—5% carbon dioxide humidified atmosphere at 37°C. The medium used for routine subcultivation was Dulbecco’s...
Modified Eagle’s Medium (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Midazolam (Dormicum®; Roche Ltd., Basel, Switzerland) was kindly donated from regional distributor of Roche Company (Seoul, Korea). Midazolam was dissolved with 0.9% NaCl solution; 0.9% NaCl solution was used as vehicle for treatment of midazolam.16 For the induction of macrophage activation, a bacterial endotoxin lipopolysaccharide (300 ng/ml) was used. Lipopolysaccharide from Escherichia coli serotype O26:B6 was purchased from Sigma (St. Louis, MO). Horseradish peroxidase–labeled anti-rabbit and anti-mouse antibodies were from Amersham (Buckinghamshire, United Kingdom). Human TNF-α, β-actin, reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma. Antibodies used for Western blotting were anti-iNOS, IkB-α (Santa Cruz, CA), anti-COX-2 (BD Biosciences, San Diego, CA), anti–phospho-p38, and anti–extracellular signal-regulated kinase (Cell Signaling, Danvers, MA).

**Western Blot Analysis**

For Western blot analysis, RAW264.7 cells were harvested in 100 μl lysis buffer containing 20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 1 mM β-glycerophosphate, 4 mM Na pyrophosphate, 5 mM NaF, 1% Triton X-100, and protease inhibitor cocktail. The lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was collected. Protein (40 μg) was separated by SDS-PAGE (9% for iNOS; 10% for COX-2; 12.5% for IkB-α, phospho-p38, and phospho-extra cellular signal-regulated kinase) and was electro transferred onto polyvinylidene difluoride membranes. After blocking with 5% skim milk for 2 h at room temperature, blots were incubated for overnight at 4°C with specific primary antibody (1:1,000), and subsequent detection with horseradish peroxidase–conjugated secondary antibody was performed. Blots were developed for visualization using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

**Transfection and Reporter Assays**

To study the effect of midazolam on nuclear factor-κB (NF-κB) activity, reporter assay was performed in the NF-κB-transfected human embryonic kidney 293 cells. Because of low transfection efficiency of RAW264.7, we chose human embryonic kidney 293 cells, which were widely used in transfection assay. TNF-α was used to stimulate NF-κB activation, instead of lipopolysaccharide, because Toll-like receptors for lipopolysaccharide are not expressed in human embryonic kidney 293 cells and TNF-α can be produced by lipopolysaccharide in macrophage. For transfections, 5 × 10⁴ cells in six-well plates were transfected with 1 μg NF-κB-luciferase or Renilla luciferase, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For cotransfection experiments, equimolar amounts of each plasmid were used for a total of 1 μg DNA per six wells. One day after transfection, cells were stimulated with TNF-α (15 ng/ml) in the presence or absence of midazolam for 8 h, at which time luciferase production was measured. The cells were harvested, and a dual luciferase assay was conducted according to the manufacturer’s suggestions (Promega, Madison, WI). Luciferase activities were measured using a Luminoskan Ascent (Thermo Labsystems, Waltham, MA).

**Immunofluorescent Staining**

The effect of midazolam on the NF-κB p65 translocation induced by lipopolysaccharide was measured with immunofluorescent staining of RAW264.7 cells. Because NF-κB translocation occurs within 30 min after treatment with lipopolysaccharides, RAW264.7 cells were treated with lipopolysaccharide (300 ng/ml) for 30 min in the presence or absence of midazolam. For immunofluorescent staining, RAW264.7 cells were grown on glass coverslips. After exposure to midazolam or lipopolysaccharide, cells were fixed with 1% glutaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 2% bovine serum albumin for 1 h. Coverslips were then incubated overnight at 4°C in rabbit anti-p65 (1:100) primary antibody in 1% bovine serum albumin. Cells were then washed and incubated in fluorescein isothiocyanate–labeled secondary antibodies for 1 h. Cells were then washed again and stained with 5 μg/ml propidium iodide to visualize the nucleus for 10 min, followed by further washes. Coverslips were mounted on microscope slides, and fluorescence was visualized with a Zeiss confocal microscope (Oberkochen, Germany).

**Measurement of Superoxide Production**

To study the effect of midazolam on superoxide production induced by lipopolysaccharide, RAW264.7 cells were incubated with lipopolysaccharide for 18 h in the presence or absence of midazolam. Lucigenin-enhanced chemiluminescence assay was performed to analyze the level of superoxide production as previously reported.17-19 Lucigenin (bis-N-methylacridinium nitrate) luminesces specifically in the presence of superoxide. In brief, RAW264.7 (1 × 10⁵ cells) was transferred into scintillation vials containing Krebs-HEPES buffer (100 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄, 1.03 mM K₂HPO₄, 25 mM NaHCO₃, 20 mM Na-HEPES, pH 7.4) with 5 μM lucigenin. The chemiluminescence, which occurred over the ensuing 2 min in response to the addition of 100 μM NADPH, was recorded. The emitted light units, after subtracting a blank, were used as a
untreated cells for iNOS.

in a dose-dependent manner (approximately 3–30 μM) lipopolysaccharide-induced COX-2 and iNOS expression. Pretreatment with midazolam suppressed iNOS and COX-2 expression were measured by Western blot. As shown in figure 1, COX-2 and iNOS were not detected in unstimulated cells. Lipopolysaccharide induced COX-2 and iNOS expression were measured with Western blot.

**Results**

**Midazolam Suppressed Lipopolysaccharide-induced iNOS and COX-2 Expression**

First, we investigated whether midazolam affects the expression of proinflammatory mediators in macrophages. After pretreatment with midazolam at approximately 3–30 μM, cells were exposed to 300 ng/ml lipopolysaccharide for 18 h. The levels of COX-2 and iNOS expression were measured by Western blot. As shown in figure 1, COX-2 and iNOS were not detected in unstimulated cells. Lipopolysaccharide induced COX-2 and iNOS expression. Pretreatment with midazolam suppressed lipopolysaccharide-induced COX-2 and iNOS expression in a dose-dependent manner (approximately 3–30 μM). High concentrations of midazolam (30 μM) completely abrogated the lipopolysaccharide-induced COX-2 and iNOS expression in the RAW264.7 cells.

**Midazolam Suppressed NF-κB Activity**

Next, to determine whether midazolam can directly inhibit degradation of IκB, the level of IκB-α protein was assessed after exposure of lipopolysaccharide for 30 min in RAW264.7 cells. Lipopolysaccharide caused a degradation of IκB-α. However, pretreatment with midazolam significantly suppressed the degradation of IκB-α, suggesting that midazolam inhibits NF-κB activation by blocking lipopolysaccharide-induced IκB-α degradation (fig. 2A).

It has been reported that NF-κB regulates COX-220 and iNOS21 transcription by binding to cis-elements in the promoter of these genes. To confirm this, we further examined the effect of midazolam on NF-κB luciferase activity. As shown as figure 2B, midazolam completely suppressed TNF-α-induced increase in luciferase activity. Our data indicate that midazolam inhibits NF-κB activation by blocking lipopolysaccharide-induced IκB-α degradation and/or inhibition of NF-κB binding to promoter elements.

After lipopolysaccharide exposure, translocation of NF-κB p50/p65 heterodimers to the nucleus is required for NF-κB-mediated transcription. To explore the effect of midazolam on NF-κB translocation, we examined the effect of midazolam on p65 nuclear translocation in lipopolysaccharide-stimulated RAW264.7 macrophages. As shown in figure 2C, treatment of RAW cells with lipopolysaccharide induced a marked translocation of NF-κB p65 subunit into the nucleus. Application of midazolam blocked this translocation.

**Midazolam Inhibits Phosphorylation of p38 MAPK**

To examine whether midazolam inhibits mitogen-activated protein kinase (MAPK) activation induced by lipopolysaccharide, we investigated the level of p38 MAPK phosphorylation in midazolam-treated macrophage cells. Phosphorylation of p38 MAPK was induced by lipopolysaccharide at 30 min and then recovered to basal levels within 50 min. Treatment with midazolam completely suppressed p38 activation induced by lipopolysaccharide (fig. 3). In contrast, midazolam did not suppress extracellular signal-regulated kinase activation induced by lipopolysaccharide.

**Midazolam Suppressed Reactive Oxygen Species Production**

To study whether midazolam inhibits superoxide production, we assessed NADPH-driven superoxide production. Lipopolysaccharide caused NADPH-driven superoxide production in RAW264.7 cells as shown in figure 4. Although midazolam did not affect basal NADPH-driven superoxide production, it markedly suppressed lipopolysaccharide-induced superoxide production.
The current study is the first to present molecular evidence that midazolam inhibits proinflammatory mediators in lipopolysaccharide-activated RAW264.7 macrophage cells by blocking p38 MAPK and/or NF-κB-mediated transcriptional regulation.

Midazolam was used in a vehicle solution consisting of 0.9% NaCl. In preliminary tests, dilution of midazolam in 0.9% NaCl did not affect the solubility of midazolam. In addition, treatment of cells with 0.9% NaCl did not influence lipopolysaccharide-induced COX-2 expression (data not shown). Therefore, we used 0.9% NaCl solution as a vehicle in control experiments.

Lipopolysaccharide, the major outer membrane in gram-negative bacteria, activates a variety of the mammalian cell types, including monocytes/macrophages and endothelial cells, and contributes to systemic changes in septic shock. Lipopolysaccharide has been shown to initiate intracellular signaling pathways that lead to the activation of NF-κB and MAPKs. Activation of NF-κB involves the phosphorylation of IκB on serine 32 and 36 and sequent ubiquitination and degradation of IκB and concomitant release and translocation of NF-κB.

Midazolam in the range of approximately 3 to 30 μM...
suppressed iNOS and COX-2 expression, which are known as proinflammatory mediators. Plasma concentrations of benzodiazepines used clinically are approximately between 0.1 and 50 $\mu$M. These data suggest that clinically administered midazolam has antiinflammatory actions through suppression of proinflammatory mediators in macrophage cells. Peripheral-type benzodiazepine binding sites exist on mouse macrophages. Therefore, our data suggest that midazolam, via peripheral-type benzodiazepine binding sites, may exert an antiinflammatory effect in macrophages.

The transcriptional factor NF-κB plays a crucial role in the inflammatory process. NF-κB activation can lead to transcription of proinflammatory proteins, including iNOS and COX-2. When administered to lipopolysaccharide-stimulated macrophages, midazolam inhibited NF-κB activation via suppression of IkB-α degradation and NF-κB translocation. Therefore, the antiinflammatory property of midazolam is presumably through inhibition of NF-κB activation in macrophages.

The p38MAPK pathway is known to be involved in cytokine production in the cells of the monocyte-macrophage lineage. In this study, midazolam inhibited p38 activation induced by lipopolysaccharide. MAPKs, including c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38, have been reported to play a critical role in lipopolysaccharide-mediated COX-2 and iNOS expression. In particular, it is known that inhibition of p38MAPK prevents proinflammatory cytokine production via inhibition of the promoter activities of iNOS and COX-2. Therefore, inhibition of p38 MAPK by midazolam may contribute to the reduction in iNOS and COX-2 expression. Moreover, recent data have established an antiinflammatory effect of a p38 MAPK inhibitor during human endotoxemia, thus providing hope for the future use of p38 MAPK inhibitors in patients with inflammatory diseases.

Macrophages are key components of the inflammatory response to tissue injury. Dramatic elevation of reactive oxygen species (ROS) is produced by inflammatory stimuli such as lipopolysaccharide and proinflammation cytokines (such as TNF-α or IL-1β) and brings about the changes of multiple cellular functions such as DNA synthesis, transcription factor activation, gene expression, and proliferation. It has been also shown that the MAPK pathway and MAPK-mediated proinflammatory mediator production are redox dependent, at least in part, on NF-κB/ROS-sensitive mechanisms. In the current study, p38 MAPK activation was blocked by midazolam in macrophage cells. In addition, midazolam suppressed superoxide production induced by lipopolysaccharide in macrophages as shown in figure 4. Based on these findings, our data suggest that inhibition of p38 MAPK might be due to decreased superoxide production induced by midazolam. The likely mechanism that accounts for the antiinflammatory action of midazolam involves the inhibition of lipopolysaccharide-induced superoxide production. Our data shows that ROS production in the basal state was not significantly inhibited by midazolam, but increased ROS by lipopolysaccharide was significantly inhibited by pretreatment with midazolam, suggesting that midazolam may specifically inhibit lipopolysaccharide-induced signaling pathway in macrophage. The excessive production of ROS by the activation of macrophages plays a pivotal role in the pathogenesis of inflammation and tissue injury. Therefore, suppression of ROS production by midazolam may be beneficial to oxidative stress–induced organ dysfunction.

There are some limitations to our study. Macrophages play a central role in the antibacterial host defense mechanism as a component of nonspecific cell-mediated immunity. Therefore, reduction of macrophage function is thought to act advantageously or disadvantageously. However, limited data on the attenuating effect of midazolam on the acute organ injury are available. Therefore, further studies are required to verify this beneficial effect. Another limitation in this experiment is the pre-treatment of midazolam before exposure of lipopolysaccharide. Even if midazolam inhibits lipopolysaccharide-induced ROS and NF-κB activation, the effect of posttreatment of midazolam after exposure of lipopolysaccharide, like clinical events such as in septic patients, needs further evaluation. As established by the inhibitory effect of midazolam on the proinflammatory mediators in this study, use of midazolam in the condition of ongoing inflammation process is carefully recommended.

Taken together, our data suggest that midazolam can modulate the inflammatory response by redox-dependent mechanisms in macrophages. Antiinflammatory action of midazolam might be related to the suppression of lipopolysaccharide-induced COX-2 and iNOS in macrophages.

References