Anesthesiology 2009; 110:106–12

Midazolam Inhibits Tumor Necrosis Factor-α–induced Endothelial Activation

Involvement of the Peripheral Benzodiazepine Receptor

Hee Kyoung Joo, B.Sc., Sae Cheol Oh, M.D., Ph.D.,† Eun Jung Cho, M.Sc.,* Kyoung Sook Park, M.Sc.,‡ Ji Young Lee, B.Sc.,* Eun Ji Lee, B.Sc.,* Sang Ki Lee, Ph.D.,§ Hyo Shin Kim, Ph.D.,§ Jin Bong Park, D. V. M., Ph.D.,# Byeong Hwa Jeon, M.D., Ph.D.#*

Background: **Midazolam is widely used as an intravenous sedative. However, the role of midazolam on vascular endothelial activation is still unknown. The present study explores the action of midazolam on endothelial activation and its role to peripheral benzodiazepine receptor (PBR) in cultured human umbilical vein endothelial cells.**

Methods: **Intracellular localization of PBR in human umbilical vein endothelial cells was visualized with immunofluorescent staining. Monocyte adhesion and vascular cell adhesion molecule-1 expression were measured with monocyte adhesion assay and Western blot analysis. Involvement of PBR was assessed by using specific antagonists and small interfering RNA against PBR.**

Results: **PBR was localized in the mitochondria of human umbilical vein endothelial cells. Midazolam significantly inhibited tumor necrosis factor-α–induced vascular cell adhesion molecule-1 and monocyte adhesion in a dose-dependent manner (1–30 μM). The midazolam-mediated suppression on the tumor necrosis factor-α–induced vascular cell adhesion molecule-1 expression and monocyte adhesion were inhibited by the pretreatment of PK11195 and not inhibited by the flumazenil. Transfection of small interfering RNA for PBR decreased the expression of PBR (18 kDa) in human umbilical vein endothelial cells. Midazolam-mediated suppression on the tumor necrosis factor-α–induced vascular cell adhesion molecule-1 expression was abrogated by the transfection of small interfering RNA for PBR.**

Conclusion: **These results suggest that midazolam has an inhibitory action on the endothelial activation and that its action is related to the activation of peripheral benzodiazepine receptor localized in mitochondria of the endothelial cells.**

VASCULAR inflammation is the primary cause of cardiovascular diseases such as atherosclerosis.[1] The expression of vascular adhesion molecules and monocyte adhesion to the vascular endothelial cells are the crucial events in the pathologic process of endothelial activation.[2]

In contrast to central-type benzodiazepine receptors (CBR), the peripheral benzodiazepine receptors (PBR) have been discovered as benzodiazepine-binding sites outside the central nervous system.[3] PBR abundant in the cardiovascular system[4] has mainly been found on the outer mitochondrial membrane of cells.[5] The role of PBR and its subcellular localization in the endothelial cells is not clear.

Among the benzodiazepines, midazolam, water-soluble benzodiazepine,[6] is the most widely used anxiolytic and sedative drug for short procedures and in intensive care. Midazolam is known as a mixed-type agonist of benzodiazepine receptors. In addition to the central neuroinhibitory action of midazolam, it also interferes the synthesis of nitric oxide and tumor necrosis factor-α (TNF-α) generated by activated microglial cells, blood monocytes, and mast cells,[7–9] suggesting an inhibitory action on proinflammatory mediators. We have previously reported that midazolam has antiinflammatory actions through the suppression of proinflammatory mediators and superoxide production in macrophage cells.[10]

However, the role of midazolam in the endothelial cell activation is still unknown. In this study, we hypothesize that midazolam serves to suppress endothelial cells activation *via* activation of PBRs in endothelial cells. Therefore, we investigated the action of midazolam and the role of PBR in the endothelial cells activation in the cultured human endothelial cells.

Materials and Methods

Cell Culture and Drugs

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Cambrex, MD), and they were grown and maintained in endothelial growth medium. Cells were used between passages 3 and 6. U937 cell lines were obtained from American type culture collection (Manassas, VA). Midazolam was purchased from Bukwang Company (Seoul, Korea). Midazolam was dissolved in 0.9% NaCl solution that was used as vehicle for treatment of midazolam.[10,11] For the induction of endothelial cell activation, human TNF-α (15 ng/ml) was used. Anti-VCAM-1 and anti-β-actin were

* Postgraduate Student, § Research Professor, # Associate Professor, Department of Physiology, College of Medicine, Chungnam National University, Daejeon, Korea. † Assistant Professor, Department of Anesthesia and Pain Medicine, St. Mary's Hospital, Daejeon, Korea. ‡ Research Scientist, BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea.

Received from the Department of Physiology, College of Medicine, Chungnam National University, Daejeon, Korea; Department of Anesthesia and Pain Medicine, St. Mary's Hospital, Daejeon, Korea.; and BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. Submitted for publication June 1, 2008. Accepted September 25, 2008. Support was provided by Korea Science & Engineering Foundation through the Infection Signaling Network Research Center (R13-2007-020-01000-0, Daejeon) and Korea Science & Engineering Foundation (R01-2007-000-10974-0, Daejeon). Presented in part at the Arteriosclerosis, Thrombosis, and Vascular Biology Annual Conference, Atlanta, GA, April 16–18, 2008. H. K. Joo and S. C. Oh contributed equally to this article.

Address correspondence to Dr. Jeon, Department of Physiology, College of Medicine, Chungnam National University, 6 Munhwa-dong, Jung-gu, Daejeon, 301-131 Korea. bhjeon@cnu.ac.kr. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PBR was from Novus Biologicals. Horseradish peroxidase-labeled anti-rabbit antibodies were from Amersham (Buckinghamshire, United Kingdom). Human TNF-α, PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide, and flumazenil were purchased from Sigma (St. Louis, MO). PK11195 was dissolved with ethanol as a vehicle and then diluted in the endothelial growth medium-2 (Cambrex, MD). Maximal concentration of vehicle (ethanol) for PK11195 was 0.01%. To inhibit benzodiazepine receptors, the endothelial cells were pretreated with PK11195 and flumazenil 1 h before treatment of midazolam.

The Cloning of Human PBR(hPBR) and Subcloning into pEGFP Protein Expression Vector

The primers with the restriction enzyme linkers, sense primer: 5′-CCA AGC TTA TGG CCC CGC CCT GGG TG -3′, antisense primer: 5′-CGG AAT TCC CTC TGG CAG CCG CCG TCC-3′, were used for PCR amplification of hPBR. The nucleotide sequencing of PCR products contained an identical coding sequence of hPBR (510 bp) (Genbank accession number BC001110). PBR-EGFP plasmid was generated by inserting hPBR gene into *Hind*III and *Eco*RI sites of pEGFP-N1 (Clontech, Mountain View, CA).

Cell Transfection and Subcellular Localization Analysis with Immunofluorescent Staining

For transfection and immunofluorescent staining, 5×10^4 cells of HUVEC cells were grown on glass coverslips and then transiently transfected with 1 μg of pPBR-EGFP by using Effectene, as recommended by the manufacturer (Qiagen, Valencia, CA). 24 h after transfection, 10 nM of Mitotracker Red CNXRos (Invitrogen, Carlsbad, CA) for 1 h was used for staining mitochondria. Coverslips were mounted on microscope slides, and fluorescence signals were visualized with an Olympus confocal microscope.

siRNA Preparation and Transfection

Small interfering RNAs (siRNAs) to human PBR consisting of 21 nucleotides were synthesized from Bioneer Co. (Daejeon, Korea). The GenBank accession number for human PBR is NM_000714. siRNA duplexes with the following gene-specific sense sequences were used: PBR 579 siRNA targeting human PBR exon, 5′-CCA UGG CUG GCG UGG GGG AdTdT-3′; PBR 564 siRNA targeting human PBR exon, 5′-CGU AUG GCG GGA CAA CCA UdTdT-3′; PBR 549 siRNA targeting human PBR exon, 5′-CAC ACU CAA CUA CUG CGU A-3′; PBR 788 siRNA targeting human PBR exon, 5′-CCU GUG CUU UCU GCA UGC U-3′. The negative control siRNA (Bioneer Co) was used as control; negative control oligonucleotide template, 5′-CCUACGCCACCAAUUUCGU-3′. A single transfection of 100 nM siRNA duplexes was performed using the Lipofectamine 2000 reagent (Invitrogen). The cells were assayed for silencing later. Cells had been seeded the previous day in the endothelial basal medium-2 supplemented with 2% fetal bovine serum and endothelial growth medium-2 singlequots (Cambrex, Rutherford, NJ). The siRNA duplex was mixed with Opti-MEM. In a separate tube, the Lipofectamine 2000 reagent was mixed with Opti-MEM and incubated for 5 min at room temperature. The two solutions were combined, gently mixed by inversion, and incubated for 20 min at room temperature. The resulting siRNA-Lipofectamine 2000 was added to cells that were cultured at 30–50% confluence. Fresh endothelial growth medium-2 was added to transfected cells 5 h later. Cells were incubated at 37°C in a carbon dioxide incubator for 72 h for gene knockdown.

Monocyte-Endothelial Cell Adhesion Assay

Monocyte-endothelial cell adhesion assay was performed as described previously.[10,12] U937 cells were fluorescently labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-am.) for the quantitative adhesion assay. The U937 cells were fluorescently labeled by incubating the cells (1×10^7 cells/ml) with 1 μM BCECF-AM in RPMI-1640 medium for 30 min at 37°C and 5% CO_2. HUVECs were seeded in 24-well plates to reach confluent monolayers and pretreated with midazolam for 1 h in EGM-2 medium. Human recombinant TNF-α was added to appropriate wells (15 ng/ml) at 18 h before addition of labeled monocytes. Monocyte adhesion was quantified by measuring fluorescence with excitation (485 nm) and emission (535 nm). Wells containing HUVEC only without U937 cells were used as blanks.

Western Blot Analysis

For Western blot analysis, HUVECs were harvested with 100 μl of lysis buffer containing 20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_3, 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 (30 μg) was separated by 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. After blocking with 5% skim milk for 2 h at room temperature, blots were incubated overnight at 4°C with specific primary antibody (1:1000), and subsequent detection with horseradish peroxidase–conjugated secondary antibody was performed. Blots were developed for visualization using an enhanced chemiluminescence detection kit (Pierce Biotechnology, Rockford, IL).

Statistical Analyses

Values are expressed as the mean ± SEM. Statistical evaluation was performed using ANOVA analysis followed by *post hoc* Tukey with SPSS software (version

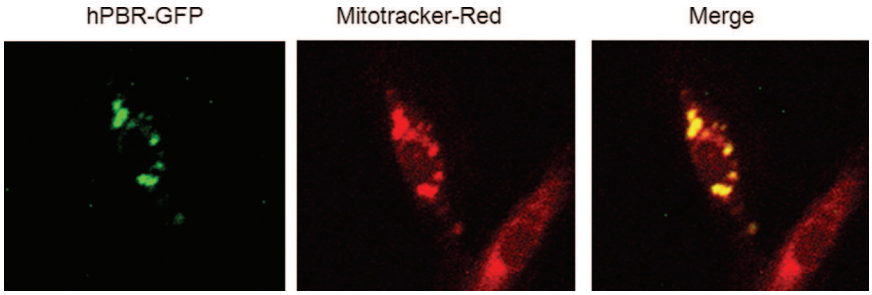

Fig. 1. Peripheral benzodiazepine receptor-enhanced green florescent protein (PBR-EGFP) expression and colocalization with mitochondria in the human umbilical vein endothelial cells. The subcellular localization of PBR-EGFP (living color of green fluorescence) and mitochondria (red fluorescence with MitoTracker Red; Invitrogen, Carlsbad, CA) was assessed by immunofluorescent staining. Fluorescence image of PBR-EGFP and mitochondria are merged in right image.

14.0, SPSS inc., Chicago, IL), with $P < 0.05$ considered significant.

Results

PBR-EGFP Expression and Subcellular Localization of PBR

We investigated the subcellular localization of PBR by immunofluorescent analysis. After transfection of the cells with pPBR-EGFP, Mitotracker red staining was performed to visualize cellular mitochondria. As shown in figure 1, both mitochondria (red fluorescence with MitoTracker Red) and PBR-EGFP (green fluorescence) presented perinuclear localization. When the images were merged, a colocalization between mitochondria and PBR was clearly observed, confirming a mitochondrial localization of PBR.

Midazolam Inhibits TNF-α–induced VCAM-1 Expression

To explore whether midazolam regulates VCAM-1 expression, we examined the effect of midazolam on the VCAM-1 expression in the TNF-α–stimulated HUVECs. After pretreatment with midazolam at 1–30 μM, the cells were exposed to TNF-α (15 ng/ml) for 18 h. The pretreatment with midazolam suppressed TNF-α-induced VCAM-1 expression in a dose-dependent manner (1–30 μM; fig. 2, A and B). Since 50% inhibition dose (ID_{50}) of midazolam on VCAM-1 is about 5 μM, this dose was used to examine the effect of antagonists on midazolam-mediated inhibition.

Midazolam Inhibits TNF-α–induced Monocyte Adhesion

VCAM-1 mediates the recruitment and adhesion of monocytes in the activated endothelial cells. After the inhibitory action of midazolam in TNF-α–induced VCAM-1 expression was confirmed, we evaluated the possible role of midazolam in TNF-α–induced monocyte adhesion to endothelial cells. Cells were pretreated with midazolam for 1 h before treatment with TNF-α. After incubation with TNF-α for 18 h, monocyte adhesion assay was performed as described in Material and Methods. As shown in figure 3, monocyte adhesion was minimal in unstimulated endothelial cells, but treatment with TNF-α resulted in a marked increase in monocyte adhesion to endothelial cells. Pretreatment with midazolam (3 to 30 μM) inhibited TNF-α–induced monocyte adhesion to endothelial cells. However, midazolam itself did not induce the monocyte adhesion, suggesting that midazolam has specific inhibitory action on monocyte adhesion induced by TNF-α to endothelial cells.

Effect of Benzodiazepine Receptor Antagonists on Midazolam-mediated Suppression of VCAM-1 Expression

Midazolam is classified as a mixed-type agonist of benzodiazepine receptors according to their affinity to the central and peripheral binding site.[13] We next studied the involvement of benzodiazepine receptors in the midazolam-mediated suppression of VCAM-1 expression. To test the involvement of benzodiazepine receptors, we used PK11195, an antagonist for the peripheral benzodiazepine receptor, and flumazenil, an antagonist for

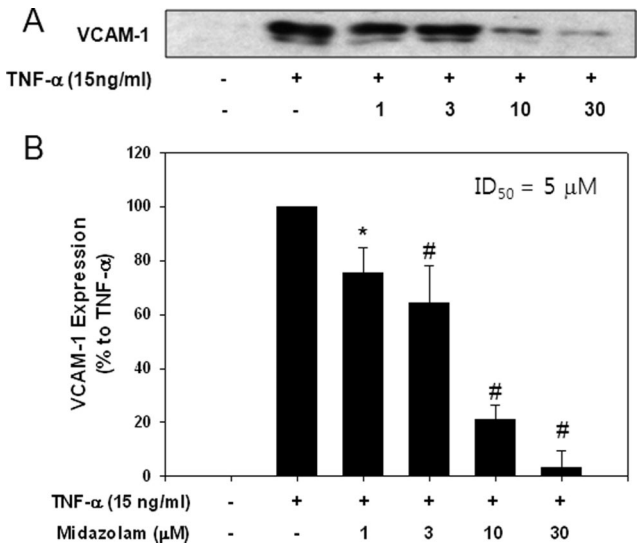

Fig. 2. Effect of midazolam on tumor necrosis factor-α (TNF-α)–induced vascular cell adhesion molecule-1 (VCAM-1) expression in the endothelial cells. Endothelial cells were incubated with an indicated concentration of midazolam for 1 h before the treatment of TNF-α. After treatment with TNF-α for 18 h, cultured cells were harvested and transferred for Western blot analysis as described in the Material and Methods section. (*A*) Typical Western blot data. (*B*) Densitometric data. Data are represented as percent densitometric value of VCAM-1 expression induced by TNF-α. Each bar shows mean ± SE (n = 6). * $P < 0.05$ and # $P < 0.01$ *versus* TNF-α alone.

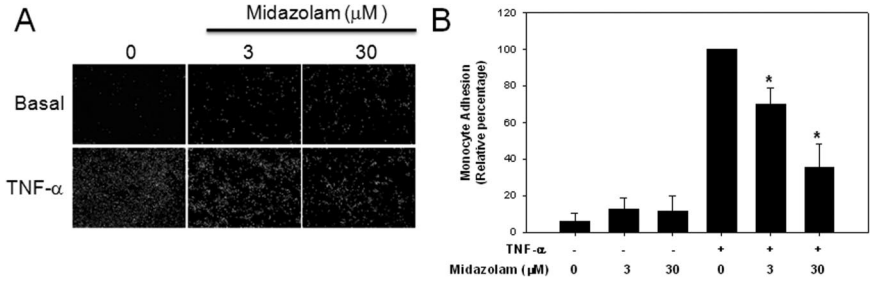

Fig. 3. Effect of midazolam on tumor necrosis factor-α (TNF-α)–induced monocyte adhesion on the endothelial cells. Midazolam was pretreated for 1 h before treatment of TNF-α (15 ng/ml). Monocyte adhesion was assessed by monocyte-endothelial cell adhesion assay as described in Material and Methods. (*A*) Typical monocyte adhesion image. (*B*) Densitometric data. Expression levels are represented as percent densitometic value of monocyte adhesion induced by TNF-α. Each bar shows mean ± SE (n = 6). * $P < 0.01$ *versus* TNF-α alone.

the benzodiazepine receptor in the CNS. As shown in figure 4, pretreatment with 10 μM flumazenil did not affect the midazolam-mediated suppression of VCAM-1 expression, suggesting that the central benzodiazepine receptor is not involved in the action of midazolam. In contrast, the pretreatment with 3 μM PK11195 significantly inhibited the midazolam-mediated suppression of VCAM-1 expression in HUVEC, suggesting the involvement of PBR in the action of midazolam.

Effect of Benzodiazepine Receptor Antagonists on Midazolam-mediated Suppression of Monocyte Adhesion

We examined whether the action of midazolam on monocyte adhesion was mediated by the PBR. To test the involvement of benzodiazepine receptors, we used PK11195 and flumazenil. As shown in figure 5, the pretreatment with 10 μM flumazenil did not reverse the action of midazolam on the monocyte adhesion induced by TNF-α. In contrast, the pretreatment with 3 μM PK11195 significantly attenuated the action of midazolam on the monocyte adhesion induced by TNF-α in HUVEC, suggesting the involvement of PBR in the action of midazolam.

Effect of Silencing PBR Expression on Midazolam-induced VCAM-1 Inhibition

We evaluated the efficiency of siRNAs (100 nM) targeting various PBR coding regions on the PBR expression in the HUVEC. Seventy-two hours of treatment with siRNA (100 nM) targeting various PBR coding regions decreased PBR protein (fig. 6A). Especially, 564 PBR siRNA significantly decreased PBR protein levels. Therefore, 564 PBR siRNA (siRNA #2) was used in the following study.

After treatment of HUVEC cells with 100 nM PBR-specific 564 siRNA (siRNA #2) for 72 h, the inhibitory

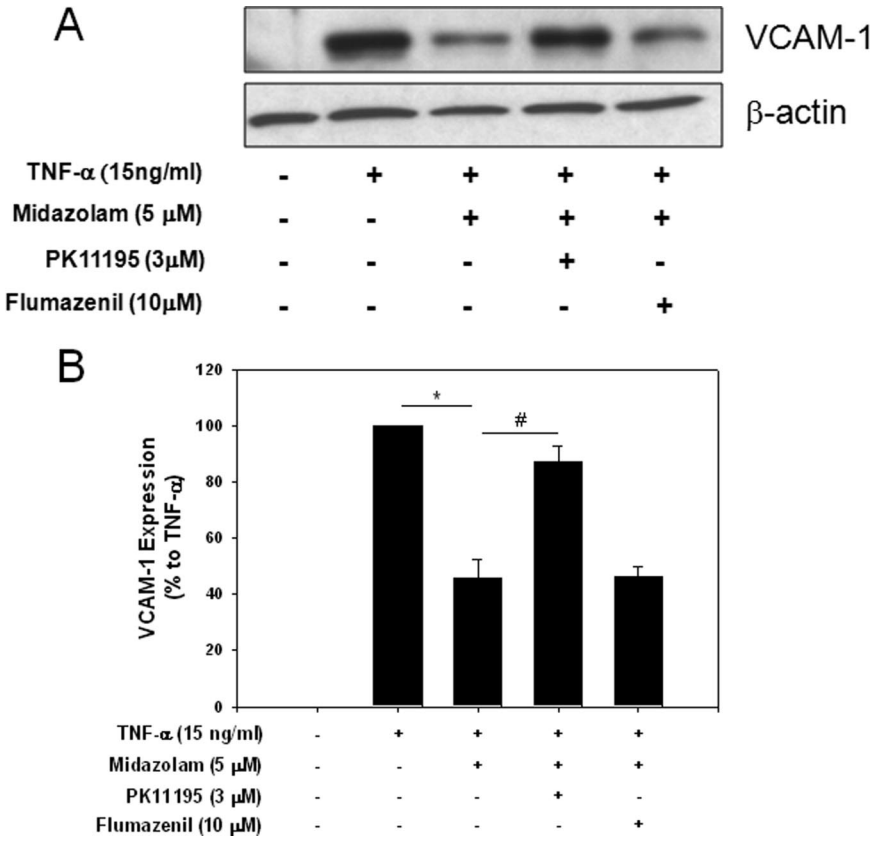

Fig. 4. Effect of benzodiazepine receptor antagonists on midazolam-mediated suppression of vascular cell adhesion molecule-1 (VCAM-1). Expression of VCAM-1 and β-actin was assessed by Western blot. HUVEC cells with or without pretreatment with 3 μM PK11195, an antagonist of peripheral benzodiazepine receptor, or 10 μM flumazenil, an antagonist of central benzodiazepine receptor, and/or 5 μM midazolam were stimulated with TNF-α for 18 h. β-actin was used as protein loading control. (*A*) Typical Western blot data. (*B*) Densitometric data. Data are represented as percent densitometric value of VCAM-1 expression induced by TNF-α. Each bar shows mean ± SE (n = 6). * $P < 0.01$ *versus* TNF-α alone, # $P < 0.01$ *versus* TNF-α in the presence of midazolam.

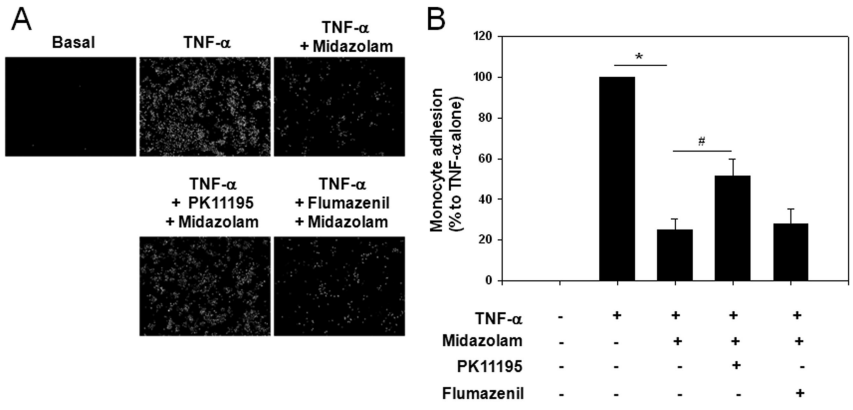

Fig. 5. Effect of benzodiazepine receptor antagonists on the action of midazolam on the monocyte adhesion. Human umbilical vein endothelial cells with or without pretreatment with 3 μM PK11195, an antagonist of peripheral benzodiazepine receptor, or 10 μM flumazenil, an antagonist of central benzodiazepine receptor, and/or 5 μM midazolam were stimulated with TNF-α for 18 h. Monocyte adhesion was assessed by monocyte–endothelial cell adhesion assay as described in Material and Methods. (*A*) Typical monocyte adhesion image. (*B*) Densitometric data. Expression levels are represented as percent densitometic value of monocyte adhesion induced by TNF-α. Each bar shows mean ± SE (n = 6). * *P* < 0.01 *versus* TNF-α alone; # *P* < 0.01 *versus* TNF-α in the presence of midazolam.

action of midazolam to TNF-α–induced VCAM-1 expression was significantly suppressed as shown in figure 6, B and C. However, PBR expressions were not affected by the treatment with midazolam and/or TNF-α. These data strongly suggested that the activation of PBR by midazolam was involved in the antiinflammatory action of midazolam in the endothelial cells.

Discussion

The present study demonstrates that midazolam has an antiinflammatory action against endothelial cell activation. The antiinflammatory action of midazolam was mediated by activating the peripheral benzodiazepine receptor in the endothelial cells. Also, the data provided here indicate that PBR plays a crucial role of antiinflammatory action in endothelial cells.

To confirm intracellular localization of PBR in the present study, we cloned the human PBR cDNA using reverse transcriptase–polymerase chain reaction in HUVECs. Isolated PBR cDNA was completely matched with GenBank accession number BC001110. The human PBR cDNA encodes for a 170–amino acid (18 kDa) protein. Isolated hPBR cDNA was then subcloned into EGFP expressed vectors to study the intracellular localization of hPBR. In 1999, PBR in the endothelial cells was first demonstrated in the dermal vascular endothelial cells.[14] The putative function of PBR is related to its intracellular localization. In the present study, we confirmed that the PBR of endothelial cells were localized in the mitochondria by using fluorescent imaging for PBR (fig. 1). Mitochondria play a central role in cell function by regulating adenosine triphosphate production and induction of cell death.[15] A previously

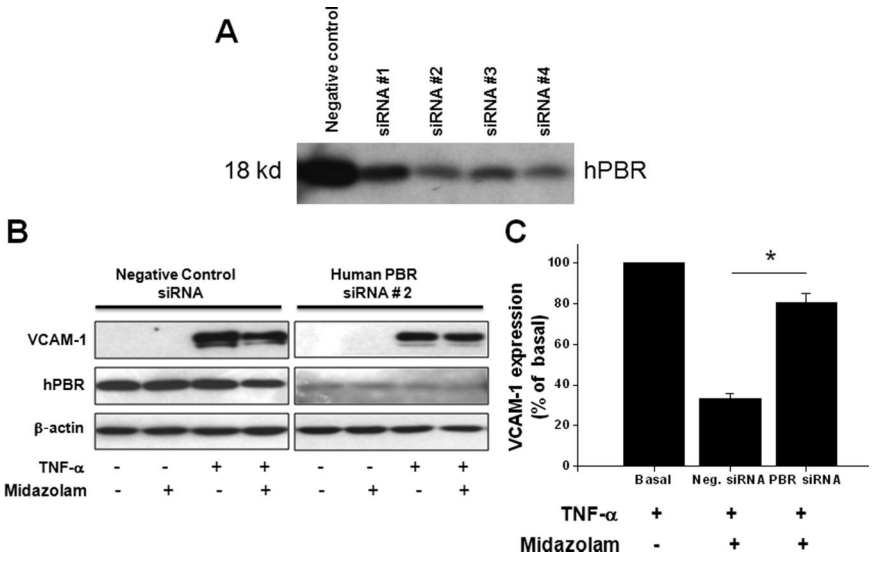

Fig. 6. Effect of siRNA targeting peripheral benzodiazepine receptor (PBR) on the action of midazolam to vascular cell adhesion molecule-1 (VCAM-1) expression in the human umbilical vein endothelial cells (HUVEC). (*A*) siRNAs targeting PBR decrease PBR proteins in the cultured HUVEC. Cells were transfected with 100 nM siRNAs targeting different coding regions of PBR or negative control siRNA and were analyzed 72 h later for PBR protein by Western blot analysis. siRNA#1, PBR 579 siRNA targeting human PBR exon; siRNA#2, PBR 564 siRNA targeting human PBR exon; siRNA #3, PBR 549 siRNA targeting human PBR exon; siRNA #4, PBR 788 siRNA targeting human PBR exon. Specific sequences of siRNA are described in Material and Methods. (*B*) Effect of PBR siRNA on inhibitory action of midazolam (5 μM) to TNF-α (15 ng/ml)–induced VCAM-1 expression. Used PBR siRNA #2 was PBR 564 siRNA targeting human PBR exon. Cells were transfected with 100 nM 564 PBR siRNAs or negative control siRNA and were then treated with TNF-α (15 ng/ml) in the presence or absence of midazolam (5 μM). Cells were analyzed 72 h later for PBR protein by Western blot. (*C*) Densitometric data. Expression levels are represented as percent densitometic value of VCAM-1 induced by TNF-α. Each bar shows mean ± SE (n = 3). * *P* < 0.05 (negative siRNA *vs.* PBR siRNA).

published report has shown that the mitochondrial localization of PBR may be involved in the inhibition of mitochondrial respiration.[16]

As a benzodiazepine derivative, midazolam is a mixed-type agonist of PBRs, and it is the most widely used anesthetic for sedation. To investigate the role of midazolam in the endothelial cell activation, we used TNF-α to induce endothelial cell activation as we have done in our previous reports.[12,17] TNF-α is known to cause monocyte adhesion, the expression of adhesion molecules, and marked oxidative stress in the endothelial cells.[12]

VCAM-1 gene encodes a cell surface sialoglycoprotein expressed by cytokine-activated endothelium. VCAM-1 protein mediates leukocyte-endothelial cell adhesion and signal transduction, and it may play a role in the development of atherosclerosis. In the present study, we found that midazolam in the range of 3–30 μM suppresses VCAM-1 and monocyte adhesion in the TNF-α–activated endothelial cells. The plasma concentrations of benzodiazepines used clinically were approximately between 0.1 and 50 μM.[7,18] These data suggest that clinically administered midazolam has antiinflammatory properties through suppression of monocyte adhesion in endothelial cells. Therefore, our data suggest that midazolam, *via* peripheral-type benzodiazepine binding sites, may exert an antiinflammatory effect on endothelial cells. Recently, it has been reported that decreased cerebral endothelial intercellular cell adhesion molecule-1 expression by midazolam may decrease post-ischemic brain inflammation and secondary brain injury.[19]

We next investigated how midazolam acts on the antiinflammatory action in the endothelial cells. To confirm whether midazolam acts on the PBR or CBR, we used the specific antagonist for PBR or CBR. PK11195 is a widely studied PBR ligand that has been classified as an antagonist of PBR.[20–22] Flumazenil is a specific and competitive antagonist at the central benzodiazepine receptor[23] that reverses the effects of benzodiazepines by competitive inhibition at the benzodiazepine binding site on the $GABA_A$ receptor. Our data show that midazolam-mediated suppression of VCAM-1 and monocyte adhesion was specifically blocked by PK11195, and not by flumazenil, in the endothelial cells. These data suggest that midazolam has an inhibitory action against vascular endothelial cells activation *via* the binding of PBR in the endothelial cells. However, midazolam-mediated suppression on monocyte adhesion was only attenuated by PK11195, suggesting that anti-adhesive action of midazolam is not limited by acting in the peripheral benzodiazepine receptor. On the basis of our knowledge, the role of CBR is still unknown in the endothelial cells; even GABA receptors were detected in the endothelial cells (data not shown). The functional role of GABA receptors in the endothelial cells needs further evaluation.

The siRNA targeting PBR was also used to evaluate the role of PBR on the action of midazolam to VCAM-1 expression in the endothelial cells. siRNA duplexes for PBR gene were transfected in the endothelial cells. Seventy-two hours after transfection of siRNA duplexes for PBR, PBR protein expression was markedly decreased as shown in the Western blot against anti-PBR (fig. 6). Midazolam-mediated VCAM-1 suppression was significantly decreased by PBR gene silencing with siRNA duplex transfection in the endothelial cells, suggesting the involvement of PBR on antiinflammatory action.

Part of the signal transduction pathway that regulates the activation of VCAM-1 expression is redox-sensitive; therefore, the compounds with antioxidant properties may have inhibitory effects on VCAM-1 expression.[12,24] A previous report has shown that midazolam inhibits superoxide production by inhibiting nicotinamide adenine dinucleotide phosphate-oxidase in macrophage.[10] Midazolam has also been found to inhibit 2,4 dinitrophenol-uncoupled mitochondrial respiration, suggesting that midazolam primarily acts as a mitochondrial electron transport inhibitor.[25] We therefore proposed that the antiinflammatory action of midazolam is mediated by the binding of PBR. The binding of PBR with midazolam would affect the mitochondrial respiration. Reduced mitochondrial respiration resulted in reduced superoxide production; therefore, mitochondrial superoxide production induced by cytokine such as TNF-α would be suppressed by the midazolam. The endothelial cell activation induced by TNF-α can be reduced by the pretreatment of midazolam.

There are some limitations to our study. First, it was only performed in cultured endothelial cells. The beneficial effect of midazolam on the *in vivo* system is limited. Therefore, further studies are required to verify this beneficial effect. Second, regarding the clinical value of midazolam, we acknowledge that monocyte adhesion may be one of several factors contributing to the vascular endothelial activation seen in vascular inflammatory disorder. However, our data are implying a beneficial effect of midazolam in the cytokine-induced vascular inflammation. Another limitation in this study is the pretreatment of midazolam before exposure to cytokine such as TNF-α. The effect of midazolam after exposure to cytokine, like clinical situations, needs further evaluation. Even though this finding implies a beneficial effect in the cytokine-induced vascular inflammation, the clinical application for the vascular inflammation is carefully recommended.

Taken together, our data suggest that midazolam plays an antiinflammatory action in vascular endothelial activation. This antiinflammatory action might be related to binding of the peripheral benzodiazepine receptor expressed in mitochondria of the endothelial cells.

References

1. Ross R: Atherosclerosis–an inflammatory disease. N Engl J Med 1999; 340: 115–26
2. Ross R: Cell biology of atherosclerosis. Annu Rev Physiol 1995; 57:791–804
3. Braestrup C, Squires RF: Specific benzodiazepine receptors in rat brain characterized by high-affinity (3H)diazepam binding. Proc Natl Acad Sci U S A 1977; 74:3805–9
4. Le Fur G, Guilloux F, Rufat P, Benavides J, Uzan A, Renault C, Dubroeucq MC, Gueremy C: Peripheral benzodiazepine binding sites: effect of PK 11195, 1-(2-chlorophenyl)-N-methyl-(1-methylpropyl)-3 isoquinolinecarboxamide. II. *In vivo* studies. Life Sci 1983; 32:1849–56
5. Veenman L, Gavish M: The peripheral-type benzodiazepine receptor and the cardiovascular system. Implications for drug development. Pharmacol Ther 2006; 110:503–24
6. Kanto JH: Midazolam: The first water-soluble benzodiazepine. Pharmacology, pharmacokinetics and efficacy in insomnia and anesthesia. Pharmacotherapy 1985; 5:138–55
7. Wilms H, Claasen J, Rohl C, Sievers J, Deuschl G, Lucius R: Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: Evidence from activated microglial cells *in vitro*. Neurobiol Dis 2003; 14:417–24
8. Taupin V, Jayais P, Descamps-Latscha B, Cazalaa JB, Barrier G, Bach JF, Zavala F: Benzodiazepine anesthesia in humans modulates the interleukin-1 beta, tumor necrosis factor-alpha and interleukin-6 responses of blood monocytes. J Neuroimmunol 1991; 35:13–9
9. Bidri M, Royer B, Averlant G, Bismuth G, Guillosson JJ, Arock M: Inhibition of mouse mast cell proliferation and proinflammatory mediator release by benzodiazepines. Immunopharmacology 1999; 43:75–86
10. Kim SN, Son SC, Lee SM, Kim CS, Yoo DG, Lee SK, Hur GM, Park JB, Jeon BH: Midazolam inhibits proinflammatory mediators in the lipopolysaccharide-activated macrophage. ANESTHESIOLOGY 2006; 105:105–10
11. Miyawaki T, Sogawa N, Maeda S, Kohjitani A, Shimada M: Effect of midazolam on interleukin-6 mRNA expression in human peripheral blood mononuclear cells in the absence of lipopolysaccharide. Cytokine 2001; 15:320–7
12. Kim CS, Son SJ, Kim EK, Kim SN, Yoo DG, Kim HS, Ryoo SW, Lee SD, Irani K, Jeon BH: Apurinic/apyrimidinic endonuclease1/redox factor-1 inhibits monocyte adhesion in endothelial cells. Cardiovasc Res 2006; 69:520–6
13. Matsumoto T, Ogata M, Koga K, Shigematsu A: Effect of peripheral benzodiazepine receptor ligands on lipopolysaccharide-induced tumor necrosis factor activity in thioglycolate-treated mice. Antimicrob Agents Chemother 1994; 38:812–6
14. Stoebner PE, Carayon P, Penarier G, Frechin N, Barneon G, Casellas P, Cano JP, Meynadier J, Meunier L: The expression of peripheral benzodiazepine receptors in human skin: The relationship with epidermal cell differentiation. Br J Dermatol 1999; 140:1010–6
15. Di Lisa F, Bernardi P: Mitochondrial function as a determinant of recovery or death in cell response to injury. Mol Cell Biochem 1998; 184:379–91
16. Hirsch JD, Beyer CF, Malkowitz L, Beer B, Blume AJ: Mitochondrial benzodiazepine receptors mediate inhibition of mitochondrial respiratory control. Mol Pharmacol 1989; 35:157–63
17. Song YJ, Lee JY, Joo HK, Kim HS, Lee SK, Lee KH, Cho CH, Park JB, Jeon BH: Tat-APE1/ref-1 protein inhibits TNF-alpha-induced endothelial cell activation. Biochem Biophys Res Commun 2008; 368:68–73
18. Bowling AC, DeLorenzo RJ: Micromolar affinity benzodiazepine receptors: Identification and characterization in central nervous system. Science 1982; 216:1247–50
19. Ghori K, Harmon D, Walsh F, Shorten G: Effect of midazolam on *in vitro* cerebral endothelial ICAM-1 expression induced by astrocyte-conditioned medium. Eur J Anaesthesiol 2006; 23:788–92
20. Bono F, Lamarche I, Prabonnaud V, Le Fur G, Herbert JM: Peripheral benzodiazepine receptor agonists exhibit potent antiapoptotic activities. Biochem Biophys Res Commun 1999; 265:457–61
21. Okaro AC, Fennell DA, Corbo M, Davidson BR, Cotter FE: Pk11195, a mitochondrial benzodiazepine receptor antagonist, reduces apoptosis threshold in Bcl-X(L) and Mcl-1 expressing human cholangiocarcinoma cells. Gut 2002; 51:556–61
22. Rao VL, Bowen KK, Rao AM, Dempsey RJ: Up-regulation of the peripheral-type benzodiazepine receptor expression and [(3)H]PK11195 binding in gerbil hippocampus after transient forebrain ischemia. J Neurosci Res 2001; 64:493–500
23. Weinbroum AA, Flaishon R, Sorkine P, Szold O, Rudick V: A risk-benefit assessment of flumazenil in the management of benzodiazepine overdose. Drug Saf 1997; 17:181–96
24. Fruebis J, Silvestre M, Shelton D, Napoli C, Palinski W: Inhibition of VCAM-1 expression in the arterial wall is shared by structurally different antioxidants that reduce early atherosclerosis in NZW rabbits. J Lipid Res 1999; 40: 1958–66
25. Colleoni M, Costa B, Gori E, Santagostino A: Biochemical characterization of the effects of the benzodiazepine, midazolam, on mitochondrial electron transfer. Pharmacol Toxicol 1996; 78:69–76