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Midazolam Stimulates Vascular Endothelial Growth Factor Release in Aortic Smooth Muscle Cells

Role of the Mitogen-activated Protein Kinase Superfamily

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Background: **Intravenous anesthetics used during perioperative periods affect the vascular signaling molecules and the vascular reactivity. Vascular endothelial growth factor (VEGF), an angiogenesis factor produced in and secreted from aortic smooth muscle cells, is a specific mitogen for vascular endothelial cells. This study investigated the effects of various intravenous anesthetics on VEGF release, and the underlying mechanism, in a rat aortic smooth muscle cell line, A10 cells.**

Methods: **Intravenous anesthetics (midazolam and propofol) were continuously administered to rats by infusion. Cultured A10 cells were stimulated by intravenous anesthetics (midazolam, propofol, and ketamine). VEGF was evaluated by immunoassay. The phosphorylation of mitogen-activated protein (MAP) kinases was evaluated by Western blotting.**

Results: **Continuous infusion of midazolam, but not propofol, increased the VEGF concentration in rat plasma. In cultured cells, midazolam stimulated VEGF release, but propofol and ketamine did not. Midazolam induced phosphorylation of p44/p42 MAP kinase and stress-activated protein kinase (SAPK)/c-*Jun N*-terminal kinase (JNK), without affecting p38 MAP kinase. PD98059 and U0126, specific inhibitors of MAP kinase kinase, significantly reduced the midazolam-stimulated release of VEGF. SP600125, a specific inhibitor of SAPK/JNK, significantly reduced midazolam-stimulated VEGF release. Applied together, PD98059 and SP600125 produced an additive reduction in midazolam-stimulated VEGF release. Moreover, a bolus injection of PD98059 truly inhibited the midazolam-increased VEGF concentration in rat plasma *in vivo*.**

Conclusions: **Midazolam, but not propofol or ketamine, stimulates VEGF release in aortic smooth muscle cells. Its effect is mediated at least in part *via* activation of p44/p42 MAP kinase and SAPK/JNK.**

VASCULAR endothelial growth factor (VEGF) acts as a mitogen specific for vascular endothelial cells, enhances vascular permeability, and promotes angiogenesis.[1] VEGF is expressed and secreted by numerous tumor and normal cells, including vascular smooth muscle cells.[1] In vascular smooth muscle cells, it has been shown that various growth factors and cytokines (such as basic fibroblast growth factor, platelet-derived growth factor, endothelin, transforming growth factor-β, and interleukin-1) stimulate VEGF production.[2–6] As for the mechanism behind this VEGF production, it has been reported that endothelin-1 and -3 each increase VEGF synthesis through activation of protein kinase C in human umbilical vein smooth muscle cells.[6] We previously reported that p38 mitogen-activated protein (MAP) kinase plays an important role in the pathway mediating the transforming growth factor-β– or vitamin D_3–induced synthesis of VEGF in an aortic smooth muscle cell line, A10 cells.[7,8] However, the precise mechanism underlying VEGF production and release in vascular smooth muscle cells is not fully understood.

Intravenous anesthetics are used during the perioperative period in critically ill patients.[9,10] Accumulating evidence suggests that they have direct effects on the cardiovascular system[9,10]; indeed, it has been reported that such anesthetics affect hemodynamics and decrease systemic blood pressure (through reductions in myocardial contractility and systemic vascular resistance),[11–15] probably *via* modulating several signaling molecules of the vascular smooth muscle cells.[16–18] Within the vascular system, vascular smooth muscle cells, which play a crucial role in the pathogenesis of atherosclerosis and hypertension,[19] are the main source of VEGF.[20] However, the effects of intravenous anesthetics on VEGF release from vascular smooth muscle cells have yet to be clarified.

In the present study, we first established that the intravenous anesthetic midazolam (but not propofol or ketamine) induces VEGF release into the plasma in rats, and we then examined the mechanism behind this VEGF release using A10 cells.

Materials and Methods

Materials

Midazolam was kindly provided by Yamanouchi Pharmacy (Tokyo, Japan). Propofol was purchased from Aldrich (Tokyo, Japan), ketamine from Sigma Chemical (St. Louis, MO), and a mouse VEGF enzyme-linked immunosorbent assay kit from R&D Systems (Tokyo, Japan). PD98059, U0126, and SP600125 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Antibodies against phosphospecific p44/p42 MAP kinase, p44/p42 MAP kinase, phosphospecific stress-activated protein kinase (SAPK)/c-*Jun N*-terminal kinase (JNK), SAPK/JNK, phosphospecific p38 MAP kinase, and p38 MAP kinase

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were all from New England BioLabs (Beverly, MA). The enhanced chemiluminescence Western blotting detection system used was from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Propofol was dissolved in ethanol. PD98059, U0126, and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect the measurements made in either the VEGF immunoassay or the Western blot analysis.

Infusion of Midazolam or Propofol in vivo

Male Sprague-Dawley rats (SLC, Sizuoka, Japan) were used. Midazolam (0.3 mg/kg, n = 5) or propofol (1 mg/kg, n = 3) was injected as a bolus *via* the tail vein, and then the right femoral artery and left femoral vein were exposed. Catheters (Natume Co. Ltd., Tokyo, Japan) were inserted into the left femoral vein (for infusion of the agent under test) and right femoral artery (for the monitoring of blood pressure and pulse rate *via* a pressure transducer [AP601G; Nihon Kohden, Tokyo, Japan]). Midazolam ($0.1\ mg \cdot kg^{-1} \cdot h^{-1}$) or propofol ($10\ mg \cdot kg^{-1} \cdot h^{-1}$) was continuously infused using an infusion pump (STC-523; Terumo Inc., Somerset, NJ) at a constant rate of 0.5 ml per hour, the infusion being started as soon as the catheter was in place and continued for the next 8 h.

In the case of experiments investigating the effect of PD98059 during prolonged midazolam infusion *in vivo*, rats were divided into two groups, a group treated with midazolam alone (n = 6) and a group treated with midazolam after a bolus injection of PD98059 at a dose of 75 μM (n = 6), according to the method of previously described.[21,22] Midazolam (0.3 mg/kg) was injected as a bolus *via* rat tail vein and the right jugular vein was exposed. Catheter was connected to the right jugular vein for the continuous infusion by an implanted osmotic pump (model 2001D, Alzet, DURECT Corp., Cupertino, CA) at a dose of $0.03\ mg \cdot kg^{-1} \cdot h^{-1}$. Osmotic pumps were incubated for 30 min in saline (37°C) before midazolam was injected by a exclusive syringe and then it was subcutaneously implanted on the back of each rat. The infusion was started at the connection of the catheter and continued to next 24 h.

Blood samples (0.3 ml) were collected into sodium citrate (3.15%, final concentration) at various time-points *via* the jugular vein. All experiments were performed in accordance with institutional guidelines (Gifu University School of Medicine, Japan).

Cell Culture

A10 cells derived from fetal rat aortic smooth muscle[23] were obtained from the American Type Culture Collection (Rockville, MD). The cells (1×10^5 or 5×10^5) were seeded into dishes (35- or 90-mm diameter) and maintained at 37°C under a humidified atmosphere of 5% carbon dioxide and 95% air in Dulbecco modified Eagle medium containing 10% fetal calf serum. After 5 days, the medium was exchanged for serum-free Dulbecco modified Eagle medium. The cells were used for experiments 48 h thereafter.

Assay for VEGF

Cultured cells were stimulated by midazolam, propofol, or ketamine in serum-free Dulbecco modified Eagle medium for the indicated periods. The conditioned medium was then collected. The VEGF concentrations in rat plasma and the above medium were measured using a VEGF enzyme-linked immunosorbent assay kit that, according to the manufacturer, recognizes rat VEGF, the method being as previously described.[24] When indicated, the cells were pretreated with PD98059, U0126, or SP600125 for 60 min prior to stimulation by midazolam.

Western Blot Analysis of p44/p42 MAP Kinase, SAPK/JNK, and p38 MAP Kinase

Cultured cells were stimulated by midazolam in serum-free Dulbecco modified Eagle medium for the indicated periods. The stimulated cells were rinsed twice with phosphate-buffered saline, then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate, 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000*g* for 10 min at 4°C. The supernatant was used for the analysis of each MAP kinase by Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli in 10% polyacrylamide gel.[25] Western blot analysis was performed as described previously[7,8] using each of the MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on x-ray film by means of the enhanced chemiluminescence Western blotting detection system. When indicated, the cells were pretreated with PD98059, U0126, or SP600125 for 60 min prior to stimulation by midazolam.

Other Methods

The absorbance of the enzyme-linked immunosorbent assay samples was measured at 450 nm using an SLT-Labinstruments EAR 340AT (Salzburg, Austria), and absorbance was correlated with concentration by means of a standard curve. Densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad, Hercules, CA).

Statistical Analysis

The data were analyzed by ANOVA, followed by the Student-Newman-Keuls test, probability values less than

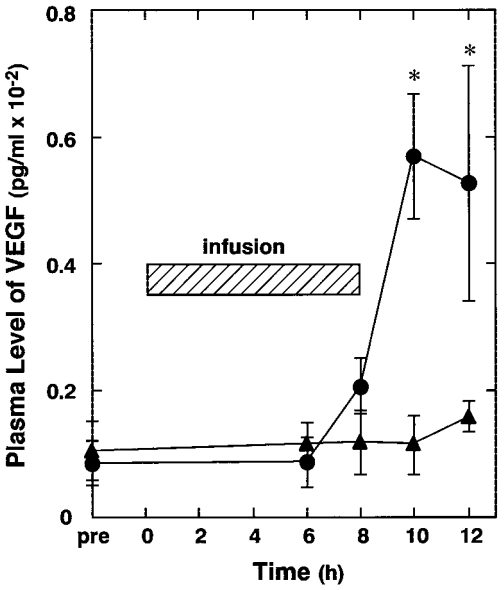

Fig. 1. Effects of midazolam and propofol on vascular endothelial growth factor (VEGF) concentration in rat plasma. Midazolam (●, 0.3 mg/kg) or propofol (▲, 10 mg/kg) was injected as a bolus and then midazolam (0.1 mg · kg^{-1} · h^{-1}) or propofol (10 mg · kg^{-1} · h^{-1}) was administered for 8 h to male Sprague–Dawley rats *via* an infusion pump. Each value represents the mean ± standard error (n = 3 propofol or 5 midazolam). *P is less than 0.05 compared with preinfusion concentration.

0.05 being considered significant. Except where otherwise noted, data are presented as the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

Results

Effects of Midazolam or Propofol on VEGF Concentration in Rat Plasma

Midazolam, not but propofol, significantly increased the concentration of VEGF in rat plasma at 10 h up to 12 h after the start of its administration (fig. 1). During the experiment, we found that neither blood pressure nor heart rate changed significantly in either group.

Effects of Midazolam, Propofol, and Ketamine on VEGF Release from A10 Cells

Next, we investigated the effects of intravenous anesthetics on VEGF release from cultured aortic smooth muscle A10 cells. Midazolam significantly stimulated VEGF release time-dependently up to 72 h (fig. 2A), the effect being significant when stimulation was for 12 h or more. The stimulatory effect of midazolam was concentration-dependent over the range 1 μM to 0.1 mM (fig. 2B), statistical significance being reached at concentrations over 30 μM. In contrast, neither propofol (1 μM to 0.1 mM) nor ketamine (1 μM to 0.1 mM) had any effect on VEGF release (fig. 2B).

Effects of Midazolam on Phosphorylation of p44/p42 MAP Kinase, SAPK/JNK, and p38 MAP Kinase in A10 Cells

To investigate the possible involvement of the MAP kinase superfamily in midazolam signaling in A10 cells, we first examined the effects of midazolam on the phosphorylation of p44/p42 MAP kinase, SAPK/JNK, and p38 MAP kinase. Stimulation by midazolam (0.1 mM) induced marked phosphorylation of p44/p42 MAP kinase and SAPK/JNK, but not of p38 MAP kinase (at any time up to 120 min) (fig. 3). The time courses of these two effects seemed to differ; the phosphorylation of p44/p42 MAP kinase peaked at between 30 and 60 min after the stimulation, then showed a slight decline. Whereas the phosphorylation of SAPK/JNK peaked at 20 min, and had declined to baseline by 60 min.

Effects of PD98059 or U0126 on Midazolam-stimulated VEGF Release from A10 Cells

In order to clarify the involvement of p44/p42 MAP kinase in midazolam-stimulated VEGF release in A10

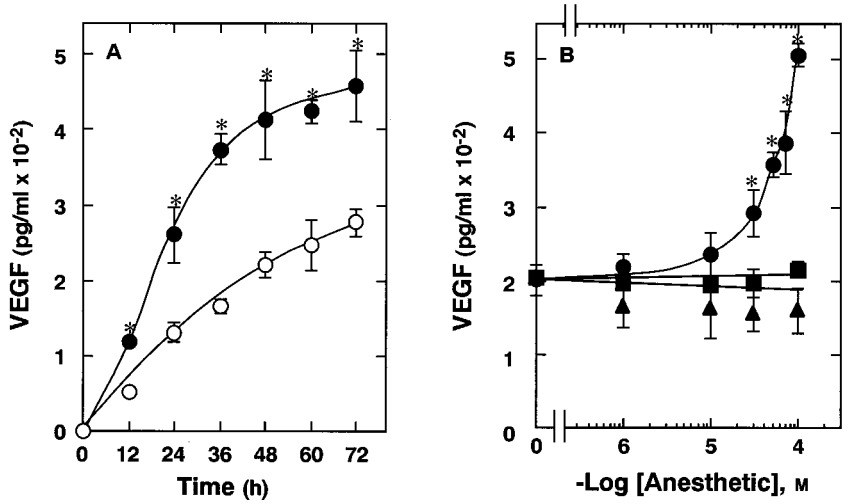

Fig. 2. Effect of midazolam on vascular endothelial growth factor (VEGF) release from A10 cells. (*A*) Time-course of VEGF release after midazolam-stimulation. Cultured cells were stimulated by 0.1 mM midazolam (●) or treated with vehicle (○) for the indicated periods. (*B*) Concentration-dependent effects of midazolam (●), propofol (▲), or ketamine (■) on VEGF release. Cultured cells were stimulated by various doses of midazolam, propofol, or ketamine for 60 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. *P is less than 0.05 compared with vehicle alone.

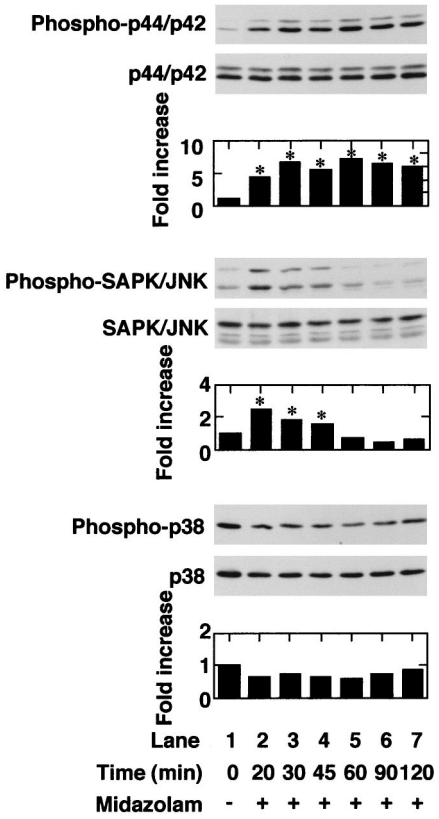

Fig. 3. Effects of midazolam on phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase, stress-activated protein kinase (SAPK)/c-*Jun N*-terminal kinase (JNK), and p38 MAP kinase in A10 cells. Cultured cells were stimulated by 0.1 mM midazolam for the indicated periods. Cell lysates were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis followed by Western blot analysis using antibodies against phosphospecific p44/p42 MAP kinase, p44/p42 MAP kinase, phosphospecific SAPK/JNK, SAPK/JNK, phosphospecific p38 MAP kinase, or p38 MAP kinase. The bars show quantitative representations of the phosphorylation of MAP kinases obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. *P is less than 0.05 compared with the value in unstimulated cells.

cells, we examined the effect of PD98059 on this VEGF release. PD98059 is a specific inhibitor of the upstream kinase (MAP kinase kinase) that activates p44/p42 MAP kinase.[26] PD98059, which by itself had little effect on the basal concentration of VEGF, suppressed the VEGF release induced by midazolam (0.1 mM). This inhibitory effect of PD98059 was concentration-dependent (0.3–50 μM), statistical significance being reached at 3 μM or more (fig. 4). In addition, U0126, another inhibitor of MAP kinase kinase,[27] which alone did not affect the basal concentration of VEGF, reduced the VEGF release. This inhibitory effect of U0126 was concentration-dependent (1–10 μM), statistical significance being seen at 3 μM or more (fig. 5).

In addition, we found that the midazolam-stimulated phosphorylation of p44/p42 MAP kinase was significantly reduced by both PD98059 and U0126 (fig. 6).

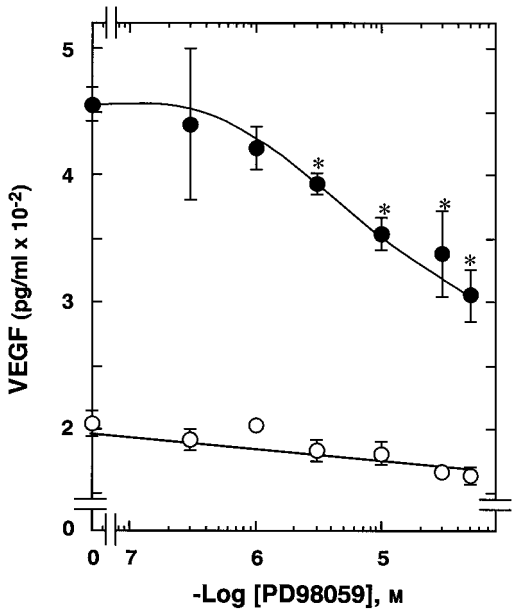

Fig. 4. Effect of PD98059 on the midazolam-induced vascular endothelial growth factor (VEGF) release from A10 cells. Cultured cells were pretreated with various concentrations of PD98059 for 60 min, then stimulated by 0.1 mM midazolam (●) or treated with vehicle (○) (each for 60 h). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. *P is less than 0.05 compared with midazolam alone.

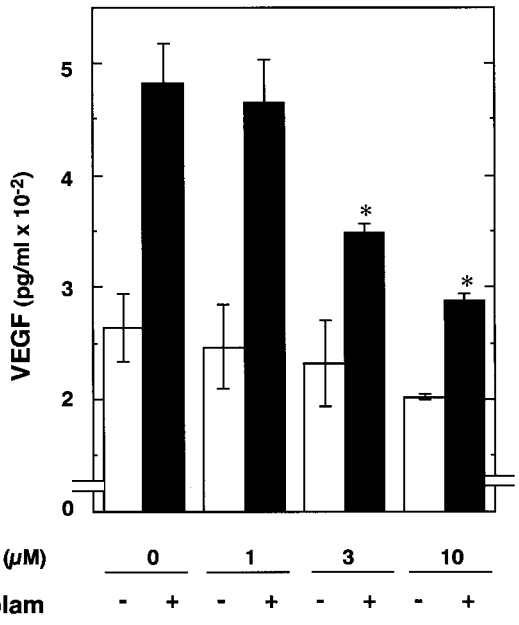

Fig. 5. Effect of U0126 on the midazolam-induced vascular endothelial growth factor (VEGF) release from A10 cells. Cultured cells were pretreated with various concentrations of U0126 for 60 min, then stimulated by 0.1 mM midazolam or treated with vehicle (each for 60 h). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. *P is less than 0.05 compared with midazolam alone.

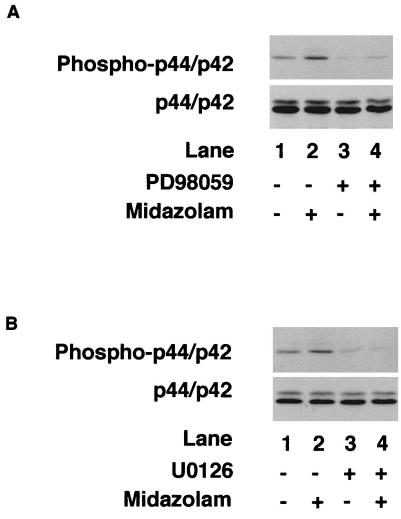

Fig. 6. Effects of PD98059 (*A*) and U0126 (*B*) on midazolam-induced phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase in A10 cells. Cultured cells were pretreated with 10 μM PD98059 (*A*) or 1 μM U0126 (*B*) for 60 min, then stimulated by 0.1 mM midazolam or treated with vehicle (each for 30 min). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using antibodies against phosphospecific p44/p42 MAP kinase or p44/p42 MAP kinase. Similar results were obtained in two other cell preparations.

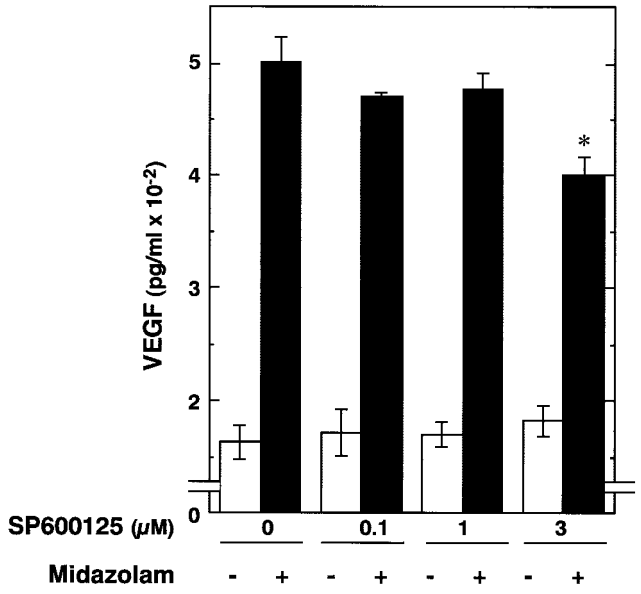

Fig. 7. Effect of SP600125 on the midazolam-induced vascular endothelial growth factor (VEGF) release from A10 cells. Cultured cells were pretreated with various concentrations of SP600125 for 60 min, then stimulated by 0.1 mM midazolam or treated with vehicle (each for 60 h). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. **P* is less than 0.05 compared with midazolam alone.

Effect of SP600125 on Midazolam-stimulated VEGF Release from A10 Cells

SP600125, a specific SAPK/JNK inhibitor,[28] which by itself had little effect on the basal concentration of VEGF, suppressed the VEGF release induced by midazolam (0.1 mM). This inhibitory effect of SP600125 was significant at 3 μM (fig. 7). In addition, SP600125 enhanced the PD98059-induced inhibition of midazolam-stimulated VEGF release (table 1).

We also found that the midazolam-stimulated phosphorylation of SAPK/JNK was significantly reduced by SP600125 (fig. 8).

Effect of PD98059 During Prolonged Midazolam Infusion in vivo

In order to further define the significant role of VEGF release induced by midazolam, we performed an additional experiment *in vivo*. We examined the effect of PD98059 *in vivo* during long-time administration of midazolam. We used 0.03 mg · kg^{-1} · h^{-1} midazolam for the stabilization of blood pressure and heart rate during 24 h. Midazolam significantly increased the concentration of VEGF in rat plasma at 12 h up to 24 h after the start of its administration during prolonged infusion (fig. 9). It is probable that the lower concentration of plasma VEGF is due to the lower concentration of midazolam. PD98059 (75 μM) significantly inhibited the VEGF concentration increased by midazolam (fig. 9).

Discussion

In the present study, our initial finding was that a continuous infusion of midazolam, but not of propofol, significantly increased the concentration of VEGF in rat plasma. The plasma concentration of VEGF first showed a tendency to increase at 8 h after the start of the infusion, with the highest concentration being detected at 2 h after the infusion ended, even though the plasma concentration of midazolam would then be decreased.[10] Since vascular smooth muscle cells are the main source

Table 1. Effects of SP600125 and PD98059 on Midazolam-induced Release of VEGF from A10 Cells

SP600125 (3 μM)	PD98059 (10 μM)	Midazolam (0.1 mM)	VEGF (pg/ml)
−	−	−	237.6 ± 11.3
−	−	+	468.1 ± 5.0*
−	+	−	211.9 ± 8.1
−	+	+	404.0 ± 21.0†
+	−	−	235.9 ± 12.5
+	−	+	404.3 ± 38.6†
+	+	−	225.2 ± 16.8
+	+	+	338.8 ± 11.6‡

Cultured cells were pretreated with 3 μM SP600125, 10 μM PD98059, and/or vehicle for 60 min, then stimulated by 0.1 mM midazolam (or treated with vehicle) for 60 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

* $P < 0.05$ compared with vehicle alone. † $P < 0.05$ compared with midazolam alone. ‡ $P < 0.05$ compared with midazolam preceded by SP600125 or PD98059.

VEGF = vascular endothelial growth factor.

Fig. 8. Effect of SP600125 on midazolam-induced phosphorylation of stress-activated protein kinase (SAPK)/c-*Jun N*-terminal kinase (JNK) in A10 cells. Cultured cells were pretreated with 3 μM SP600125 or vehicle for 60 min, then stimulated by 0.1 mM midazolam or treated with vehicle (each for 20 min). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using antibodies against phosphospecific SAPK/JNK or SAPK/JNK. Similar results were obtained in two other cell preparations.

of VEGF,[20] this finding led us to speculate that vascular smooth muscle cells may release VEGF into the blood in response to midazolam. An increase in VEGF in rat plasma was detected at 10 h after the start of midazolam administration (fig. 1). In addition, midazolam significantly increased the concentration of VEGF at 12 h up to 24 h after the start of its administration during prolonged infusion (fig. 9). Thus, it is possible that some periods are necessary for midazolam to permeate into vascular smooth muscle cells through the endothelial cells and that α-hydroxymidazolam, a main active metabolite of midazolam,[29] stimulates VEGF release in addition to midazolam in these cells. In accord with this idea, we then found that midazolam, but not propofol or ketamine,

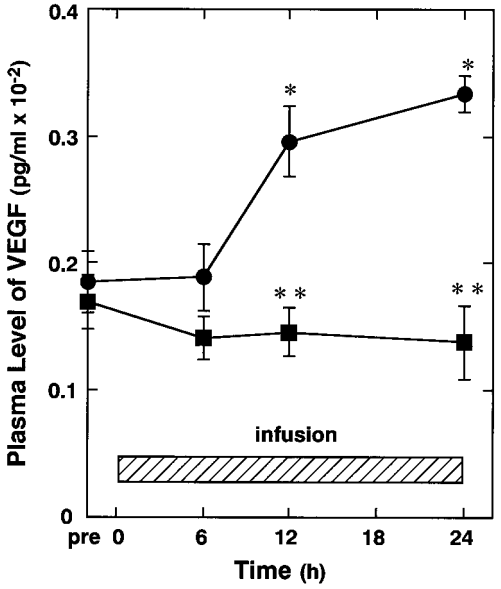

Fig. 9. Effects of PD98059 on the midazolam-increased vascular endothelial growth factor (VEGF) concentration in rat plasma. Saline (●) or 75 μM PD98059 (■) was administered. Midazolam (0.3 mg/kg) was injected as a bolus and then midazolam (0.03 mg · kg^{-1} · h^{-1}) was administered for 24 h to male Sprague-Dawley rats *via* a implanted osmotic pump. Each value represents the mean ± standard error (n = 6). **P* is less than 0.05 compared with preinfusion concentration. ***P* is less than 0.05 compared with midazolam alone.

stimulated VEGF release from cultured aortic smooth muscle A10 cells. This is the first report showing that VEGF release from vascular smooth muscle cells can be induced by midazolam, an intravenous anesthetic.

We next investigated the mechanism behind the midazolam-induced VEGF release seen in A10 cells. The MAP kinase superfamily plays an important role in transducing extracellular signaling into a cellular response, the specificity of the cellular response to a given stimulus being determined by the activation of a particular MAP kinase pathway.[30,31] Three major MAP kinases, p44/p42 MAP kinase, SAPK/JNK, and p38 MAP kinase, are generally recognized as being the central elements used by mammalian cells to transduce such diverse messages.[30,31] VEGF release is reportedly increased through activation of MAP kinases in various cells. It has been reported that anisomycin, a powerful activator of p38 MAP kinase and SAPK/JNK, increases VEGF mRNA stabilization through activation of p38 MAP kinase and SAPK/JNK in hamster fibroblast derivatives,[32] that interleukin-1β induces VEGF gene expression through p38 MAP kinase and SAPK/JNK signaling in rat myocytes,[33] and that VEGF expression is differently regulated by the p44/p42 MAP kinase and p38 MAP kinase pathways in *ras* transformed cells.[34] In the vascular system, it has been shown that both angiotensin II and platelet-derived growth factor can induce proliferation of vascular smooth muscle cells through activation of p44 MAP kinase.[35] Thus, intravenous anesthetics may affect VEGF release *via* activation of the MAP kinase superfamily in vascular smooth muscle cells.

In the present study, we demonstrated that in A10 cells, midazolam induced phosphorylation of both p44/p42 MAP kinase and SAPK/JNK, while having little effect on the phosphorylation of p38 MAP kinase. It is recognized that MAP kinases are activated by the phosphorylation of threonine and tyrosine residues by dual-specificity MAP kinase kinase.[36] Thus, our findings suggest that midazolam activates both p44/p42 MAP kinase and SAPK/JNK, but not p38 MAP kinase in aortic smooth muscle A10 cells. We next investigated whether p44/p42 MAP kinase or SAPK/JNK is involved in the pathway by which midazolam stimulates the release of VEGF in these cells. Our finding was that the midazolam-stimulated VEGF release was reduced by PD98059 and by U0126, specific inhibitors of MAP kinase kinase.[26,27] We then found that both PD98059 and U0126 truly did suppress the phosphorylation of p44/p42 MAP kinase by midazolam. Thus, these results suggested that p44/p42 MAP kinase is involved in the midazolam-induced VEGF release in A10 cells. However, the midazolam-induced VEGF release was also reduced by SP600125, a specific inhibitor of SAPK/JNK,[28] and SP600125 suppressed the midazolam-induced phosphorylation of SAPK/JNK. Taking all of these findings into account, it is most likely that activation of both p44/p42 MAP kinase and SAPK/JNK is

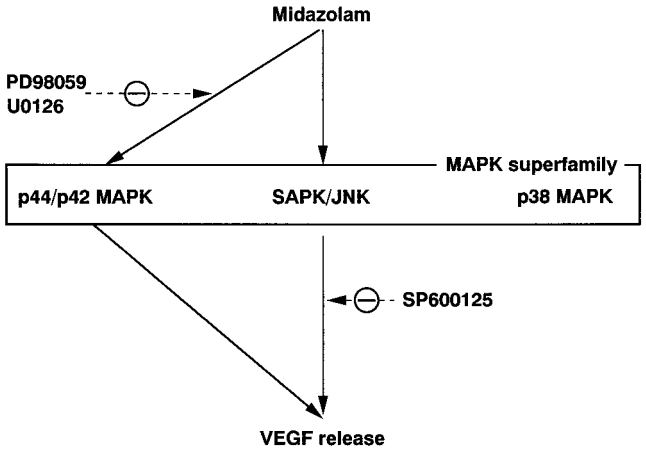

Fig. 10. Diagram of effects of midazolam on VEGF release from vascular smooth muscle cells. Dashed line = inhibitory effect; MAPK = mitogen-activated protein kinase; SAPK/JNK = stress-activated protein kinase/c-*Jun N*-terminal kinase; VEGF = vascular endothelial growth factor.

involved in midazolam-stimulated VEGF release in aortic smooth muscle A10 cells. Furthermore, we next investigated whether p44/p42 MAP kinase is involved in the pathway by which midazolam stimulates the concentration of VEGF *in vivo*. PD98059 significantly inhibited the midazolam increased VEGF concentration in rat plasma during prolonged midazolam infusion. Therefore, it is probable that midazolam increases VEGF concentration during the infusion at least in part through the activation of p44/p42 MAP kinase. The effects of midazolam on VEGF release in vascular smooth muscle cells shown in our present study is summarized in figure 10.

Previous reports of the effects of intravenous anesthetics on MAP kinases may be summarized as follows: (1) propofol inhibits the N-formyl–L-methionyl-phenylalanine-stimulated p44/p42 MAP kinase phosphorylation and chemotaxis in human neutrophils[37]; (2) when μ, δ, and κ opioid receptors are introduced into cos-7 cells, a μ, δ, or κ agonist induces activation of p44/p42 MAP kinase and SAPK/JNK, but not of p38 MAP kinase[38]; and (3) fentanyl activates p44/p42 MAP kinase in μ-expressing C6 glioma cells.[38] The present study adds to this list by being the first intimation that midazolam activates p44/p42 MAP kinase and SAPK/JNK in vascular smooth muscle cells. It has been reported that the activation of p44/p42 MAP kinase and p38 MAP kinase in vascular smooth muscle cells modulates vascular tone.[39–41] Thus, it is possible that midazolam affects systemic blood pressure through the modulation of vascular smooth muscle tone by the activation of these MAP kinases.

VEGF is a mitogen that is highly specific for vascular endothelial cells.[1] The expression of VEGF is potentiated by hypoxia, by activated oncogenes, and by a variety of cytokines.[1] VEGF induces endothelial proliferation, promotes cell migration, and inhibits apoptosis.[1] *In vivo*, VEGF induces angiogenesis as well as permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis,[1] while deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis.[1] VEGF-induced angiogenesis has also been found to play an important role both in the etiology of several diseases associated with abnormal angiogenesis and in wound repair.[1] Thus, inhibition of VEGF signaling suppresses the development of many types of tumors.[1] On the other hand, the angiogenic properties of VEGF have recently been exploited to induce *in vivo* angiogenesis for the treatment of diseases associated with an impaired blood supply,[1] and VEGF is effective for the treatment of both limb and myocardial ischemia.[42–44] Intravenous anesthetics are administered to patients with a variety of complications. The present results show that midazolam stimulates VEGF release in vascular smooth muscle A10 cells and that propofol and ketamine have no such effect. For midazolam, the therapeutic range of plasma concentrations has been reported to be 50–150 ng/ml in patients receiving a continuous intravenous infusion,[10] with the free concentration being estimated to be 13.8–41.4 nM. The effect of midazolam on A10 cells observed in the present study was obtained at concentrations higher than those used clinically. However, we demonstrated that clinically used doses of midazolam increased the plasma VEGF concentration in rats *in vivo*. Therefore, it is probable that the difference was related to the experimental conditions between *in vivo* and *in vitro*. However, further investigations will be necessary to clarify the clinical justification for its use in other patients, particularly in those in whom enhanced angiogenesis could cause problems.

In conclusion, midazolam, but not propofol or ketamine, stimulates VEGF release from rat aortic smooth muscle cells, an effect that seems to be mediated, at least, in part *via* activation of p44/p42 MAP kinase and SAPK/JNK.

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